OXYTETRACYCLINE RESIDUES AND ANTIMICROBIAL DRUG RESISTANT *ESCHERICHIA COLI* IN RAW MILK FROM DAIRY FARMS IN KANO STATE, NIGERIA

BY

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DEPARTMENT OF VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE,

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NIGERIA

SEPTEMBER, 2014

DECLARATION

I declare that the work in this Thesis entitled **"Oxytetracycline Residues and Antimicrobial Drug Resistant *Escherichia coli* in Raw Cow Milk from Dairy Farms in Kano State, Nigeria"** has been carried out by me in the Department of Veterinary Public and Preventive Medicine, Ahmadu Bello University Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

Muhammad Sanusi YUSUF _____ _____

Signature Date

CERTIFICATION

This thesis entitled**, "Oxytetracycline Residues and Antimicrobial Drug Resistant *Escherichia coli* in Raw Milk from Dairy Farms in Kano State, Nigeria"** by Muhammad Sanusi YUSUF, meets the regulations governing the award of the degree of Master of Science of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

The use of veterinary drugs in food animals may result in trace quantities of the drugs or their metabolites as residues in food. The aim of this study was to determine the occurrence and concurrence of oxytetracycline residues and antimicrobial drug resistant *Escherichia coli* in raw milk from some commercial dairy farms in Kano State. Eighteen (18%) percent of the total registered farms (54 farms) in the State were sampled using a purposive sampling technique. Twenty five percent (25%) occurrence of antimicrobial residues in milk was observed, of which 93.7% of them were tetracyclines. The distribution of antimicrobial residues in milk based on farm type indicated antimicrobial residue was highest in the conventional farms. There was a statistically significant ($P<0.05$) association in the premitest results between detection of antimicrobial residues and the type of farm. The conventional farms had 36.51% (46/126) antimicrobial residues in which 89.13% were tetracycline residues while the cooperatives had 17.65% (33/187) antimicrobial residues of which 100% were tetracycline residues. There was a statistically significant association ($p<0.05$) between detection of antimicrobial residue and the breeds of animal sampled. Occurrence of antimicrobial and tetracycline residues was highest further away from the metropolis with occurrence of 30.63% and 100%, respectively, compared to other locations. Out of 70 milk samples that were collected from within the metropolis, 19 (27.14%) had antimicrobial residues of which 14 (73.68%) were tetracycline residues. Eleven (13.25%) of the 83 samples collected from Local Governments Areas bordering Kano metropolis contained antimicrobial residues of which 100% of them are tetracycline residues. There was a statistically significant association ($P< 0.05$) in the distribution of residues between the different locations. Fifteen *E. coli* (4.8%) were isolated from the 313 milk samples collected and tested in which three (3) were from residue positive

milk and twelve (12) were from residue negative milk. Out of the four moderately resistant *E. coli* that were tested, 3 (75%) of them were from residue positive milk while 1 (25%) was from residue negative milk. Ten (90.91%) of the Multi-Drug resistant *E. coli* were from residue negative milk samples. None of the *E. coli* isolates was resistant to any of the quinolones tested, (nalidixic acid and ciprofloxacin) 13.3% were resistant to chloramphenicol (a synthetic antibiotic) and cefixime (a cephalosporin) while 33.3% were resistant to amoxicillin+clavulanic acid (a synthetic penicillin) and co-trimoxazole (a sulfa drug). About 26.7% and 40% of the isolates were resistant to gentamicin and kanamycin (aminoglycosides), respectively, while resistance to tetracycline was 46.7%. However, all of the isolates were resistant to Ampicillin (a synthetic penicillin). Result from the PCR showed that one of the isolates was carrying *tet* M resistance gene while six (40%) others were carrying *tet* A resistance gene. This study revealed the occurrence of antimicrobial residues in milk samples from commercial dairy in Kano State, Nigeria. The study further revealed that the *E. coli* isolates carry *tet* resistant genes. It is recommended that farmers should avoid the indiscriminate use of antibiotics in production, and they should observe drug withdrawal periods before milking their cows.

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ABBREVIATIONS

ABC= ATB binding cassette

ADI_s= Acceptible daily intakes

AHI= Animal health institute

AMR= Antimicrobial resistance

AOAC= Association of official analytical chemists

ATCC= American Type Culture Collection

AVMA= American veterinary medical association

CAC= Codex Alimentarius Commission

CAST= Calf antibiotic sulfa test

CVM= Center for veterinary medicine

CVMP= Committee for veterinary medicinal products

DNA= Deoxyribonucleotide

E.coli= Escherichia coli

EEC= European Economic Community

EIA= Enzyme immuno analysis

ELISA= Enzyme linked immunosorbent assay

EMB= Eosin methylene blue agar

FAO= Food and agricultural organization

FDL= Federal department of livestock

FPIA= Fluorescence polarization immunoassay

FSIS= Food safety inspection service

HPLC= High performance liquid chromatography

IDF= International dairy federation

IMVIC= Indole, methylred, vogues Proskeur, citrate

IOM= Institute of medicine

JECFA= Joint FAO/WHO expert committee on food additive

LST= Lauryl sulfate tryptose broth

MAR= Multipleantibiotic resistance index

MFS= Major facilitatorsuperfamily

MATE= Multidrug and toxic compound extrusion

MIC= Minimal inhibitory concentration

MR= Methyl red

MRL_S= Maximum residue limits

MIT= Microbial inhibition test

MUG= 4-Methylumbelliferyl-β-D-glucuronide

NAFDAC= National agency for food and drug administration and control

NAS= National academy of sciences

NCCLS= National Committee for Clinical Laboratory Standards

NOEL= No observable effect level

O157:H7= Strain of *E.coli*

OMP= Outer membrane protein

Otr= Oxytetracycline resistance

PCFIA= Particle concentration fluorescence immunoassay

PCIA= Particle concentration immunoassay

PBP= Penicillin binding protein

PCR= Polymerase chain reaction

PMF= Proton motive force

RIA= Radioimmunoassay

RND= Resistance nodulation-cell division

SMR= Small multidrug resistance

SIM= Sulfate, indole, motility test

SPSS= Statistical package for social science

STOP= Swab stick-swab test on premises

TCN= Calcium containing buffer

TLC= Thin layer chromatography

Tcr= Tetracycline resistance

Tet genes= Tetracycline resistant genes

TSI= Tripple sugar iron test

USDA= United State Department of Agriculture

USFDA= United States Food and Drug Administration

USGAO= United States General Accounting Office

VMD= Veterinary medical department

VP= Voges Proskauer

CHAPTER ONE

INTRODUCTION

In recent years, world milk production has reflected a slight upward trend. This is because people in Asia and Africa are increasingly acquiring a taste for dairy both in the form of western staples and in the form of products that provide a better match with local conditions, such as long-life dairy drinks and concentrated milk. Research shows that the total global population consumes an average of just over 100 kilograms of dairy produce per person per year, measured in terms of 'milk equivalents'. Out of this, milk consumption in Nigeria is 9.7 litres per head (Momoh, 2010).

Contamination of milk can serve as a source of health hazard to consumers of the product. A very important milk contaminant is antimicrobial residues which can lead to development of antibiotic resistant gut organisms and hypersensitivity in susceptible consumers (Cerniiglia and Kotarski, 1999). Antibiotic residues in milk are small amounts of drugs or their active metabolites which remain in milk after treating the cows (CAC, 1998). Dairy cattle that have been treated with antibiotics produce milk containing antibiotic residues for a period of time after treatment. Antibiotic residues enter the milk supply when treated cows are returned to the milking herd early or when a cow retains antibiotic residues in her system for an extraordinary length of time (CAC, 1998).

Residues of antimicrobial drugs are of global concern (Nisha, 2008) with many countries having dedicated programs for surveillance and control. The major factor identified in the occurrence of residues is improper use of veterinary medicines (Kabir *et al*., 2004) and this problem is more profound in countries like Nigeria with weak drug

regulatory structures. Several authors reported the widespread injudicious use of veterinary drugs (particularly oxytetracycline) in Nigeria due to inadequate monitoring and prescription by untrained personnel (Alhaji, 1976; Dina and Arowolo, 1991; Kabir, 1998). Levels of the drug and their metabolites may persist at unacceptable levels and consumers can be exposed to them. The presence of residues may result from failure to observe the mandatory withdrawal periods, illegal or extra-label use of drugs and incorrect dosage regimen. Unauthorized antibiotic use may result in residues of these substances in milk and tissues (Ivona and Mate, 2002).

Problems associated with antibiotic residues in milk include the risk of allergic reactions after consumption by penicillin-sensitized persons, increased resistance of pathogens towards antibiotics, and inhibition of bacterial starter cultures used in dairy production. The concerns arise mainly from the possibility that antibiotic-resistant bacteria may be transferred from animals to humans, through contact, through the environment (e.g water, manure) or through contaminated milk products (CAC, 1998). In Nigeria various studies have been conducted on drug residues deposition in milk and milk products. Oboegbulem and Fidelis (1996), Dipeolu and Alonge (2002) and Kabir *et al*. (2004) have demonstrated the presence of antibiotic residues in meat and milk.

Therapeutic use of antimicrobials may exert a selective pressure on the indigenous microflora, enhancing the potential for development or acquisition of antibiotic resistance determinants among members of the indigenous microflora, including the dominant anaerobes and facultative enteric bacteria that are more readily cultivated (Corpet, 1987; Brady and Katz, 1988; Brady *et al*., 1993; Nord, 1993). Antimicrobial resistance (AMR) refers to the resistance of a microorganism to an antimicrobial medicine to which it was previously sensitive including bacteria, viruses and parasites.

AMR is a consequence of the use, particularly injudicious use of antimicrobial medicine, and is said to develop when the microorganism mutates or acquires a resistance gene (W.H.O, 2012). Antibiotic resistance is now recognized as a major public health challenge facing global health at the beginning of the 3rd millennium in both developed and developing countries. There is higher prevalence of resistance in developing countries because of less well regulated antimicrobial policies and poor hygienic conditions and infection control practices. The spread of bacterial resistance has major implications to human health (Barbosa and Levy, 2000; W.H.O, 2006; Rossolini and Thaller, 2010).

Global spread of AMR threatens the continued effectiveness of many drugs used today (Rossolini and Thaller, 2010; Deshpande and Joshi, 2011). The global public health crisis is heightened by the fact that infectious diseases top the list for causes of death worldwide (Barbosa and Levy, 2000). AMR has become a global concern as geographic borders among countries and continents have become less distinct due to increasing global trade, expanding human and animal populations, societal advances and technological developments (Veterinary Public Health Module, 2012).

In food-producing animals, tetracyclines may be administered orally in food or drinking water, parenterally, or by intramammary infusion. Due to enterohepatic circulation, tetracycline residues may persist in the body long after administration. The levels of tetracycline residues in animal products depend on the initial dosage and the duration between drug administration and animal product collection (Gevers, 2002). The tetracyclines were one of the first groups of antimicrobial agents for which the term broad spectrum was used, because they inhibit protein synthesis of a wide range of Gram-positive and Gram-negative bacteria, atypical organisms such as *Chlamydiae*,

Mycoplasmas, Rickettsiae, and protozoan parasites. Due to the spectrum of activity, the absence of major adverse side effects, and the low production cost, tetracyclines have been widely used throughout the world in fighting infections in humans, animals, fish and plants. Given their long history of extensive use, resistance to tetracyclines has become widespread (Levy, 1992), resulting in reduced effectiveness. Nevertheless, they continue to play important roles in both human and veterinary medicine. A new generation of tetracyclines, the glycylcyclines, are specifically being developed to overcome problems of resistance to first and second generation tetracyclines (Chopra, 2001). Bacterial resistance to tetracyclines was first reported in *Shigella dysenteriae* in 1953, shortly after their discovery (Roberts, 1996). Prior to this, the majority of commensal and pathogenic bacteria were susceptible to tetracyclines, as illustrated by the finding that among 433 different members of the *Enterobacteriaceae* collected between 1917 and 1954, only 2% were tetracycline resistant (Hughes and Datta, 1983). The emergence of resistance has followed the introduction of these agents for human, animal, and agricultural use. Tetracycline resistance *(*Tcr*)* has now become widespread in both Gram-negative and Gram-positive species due to acquisition of tetracycline resistance genes *(tet* genes) located on transposons or plasmids. So far, three different bacterial strategies of Tcr have been identified, and more than 30 different genes have been reported (Gevers, 2002).

1.1 Statement of Problem

Nigeria like many sub-saharan African countries is bedeviled with many enzootic livestock and poultry diseases requiring the use of drugs, either for prevention or treatment of infectious diseases. One of the drugs that is popular in poultry and livestock production in Nigeria is oxytetracycline partly because of its field use as a

long acting preparation. In the process this drug is injudiciously used. Olatoye and Ehinmowo (2010) have reported that oxytetracycline is routinely misused and abused in Nigeria. Despite its widespread use, there are limited studies on the occurrence of its residues in dairy animals. The major supply of dairy products in Nigeria is still the Fulani traditional dairy system. It is this system that is often considered more uncontrolled than conventional dairy farms where drugs are obtained and used without veterinary guidance and observance of withdrawal period is hardly practiced predisposing the public to the dangers of veterinary drug residues. There have been a number of attempts by State and Federal interventions to organize traditional dairy system into commercial groups with limited success and even though few commercial dairy farms have emerged within major cities, there is still limited evidence of their compliance with global best practices in ensuring consumer protection since no formal milk inspection service exist in Nigeria.

Several studies have reported the occurrence and emergence of multidrug resistant strains such as *E.coli* and *Salmonella* as a result of improper and profuse use of antimicrobial drugs in animal production, a twin problem to the occurrence of residues (Neu *et al.*, 1975; Lyons *et al.*, 1980; Nair *et al.*, 1995 and Davison, 1999). This is responsible for a very large pool of resistance genes in the environment. The existence of large antibiotic resistance gene pools in food borne commensal bacteria in ready-to-eat food items suggests that humans are constantly inoculated with large numbers of antibiotic resistant bacteria through daily food intake independent of clinical antibiotic exposure. The impact of commensal bacteria on the emergence, amplification, dissemination and maintenance of antibiotic resistance gene pool has been recognized (Wang and Schaffner, 2011). There is promiscuous exchange of resistance genes

between commensals and pathogens which serve as reservoirs of resistance genes propagated in animals and transmitted to humans through food, water, sludge and manure used as fertilizers (Marshall and Levy, 2011). AMR is global in distribution and the problem is heightened in developing countries including Nigeria. There is no attempt to review in detail the situation in Nigeria, but studies carried out on diverse groups of microbes from animals, foods of animal origin and humans carried out in Nigeria have continued to reveal an alarming dimension on the prevalence of AMR microbes as 60 to 100% of the organisms displayed multidrug resistance phenotypes (Kwaga and Adesiyun, 1984; Kwaga *et al.*, 1986,1987,1988; 2004; Esona *et al.*, 2004; Whong and Kwaga, 2007; Fashae *et al.*,2010; Tafida, 2011; Ndahi, 2012).

When antibiotics are used, emergence of drug resistant microorganisms is inevitable, especially when used inappropriately (Kwaga, 2012). Therefore, antibiotic exposure is central to the emergence of antimicrobial resistant bacteria. Drug use and resistance are closely related temporally (D' Agata *et al.*, 2008; Altunsoy *et al.*, 2011; Tadesse *et al.*, 2012). The use of antibiotics in animals, contribute to the problems in humans (Kwaga, 2012). Tadesse *et al.* (2012) tested 1,729 *E. coli* isolates recovered from animals and human samples from 1950-2002 for susceptibility to 15 antimicrobial agents and detected increased resistance from 7.2% to 63.6%.

1.2 Justification

The increasing importance of milk as a major source of nutrition and protein source for humans and recent campaign by various government agencies on food security shows that there is need to evaluate the safety of the milk in our environment particularly Kano

State due to relatively high cattle population and cosmopolitan nature of the State. Also commercial dairy constitute a significant fraction of the dairy industry in the State.

Recent studies by Momoh, (2010) indicated high consumption rate of milk in Nigeria, this suggests that more people will be at risk of exposure if the milk they consume contain residue levels above the maximum residue levels (MRLs). There is paucity of information on the level of drug residues in milk consumed by the public in Kano State. This study will evaluate the tetracycline residues levels in milk and antimicrobial drug resistant *E. coli.*

Previous researches have evaluated the effects of antibiotic residues in milk and established their presence but have not demonstrated the relationship between resistance of *E. coli* and acquisition of resistance genes. This work will provide information in this regard that is important in the control of antibiotic resistance.

1.3 Aim

To determine the occurrence and concurrence of oxytetracycline residues and antimicrobial drug resistant *Escherichia coli* in raw milk from some commercial dairy farms in Kano State.

1.4 Objectives

- To determine the occurrence of oxytetracycline residues in raw cow milk samples in Kano State.
- To isolate and identify *E. coli* from the raw milk samples.
- To determine the susceptibility of the *E.coli* isolates to a panel of antimicrobials.
- To determine the presence of *tet* genes from the *E. coli* isolates.

1.5 Research Questions

- Are oxytetracycline residues present in raw cow milk samples from commercial dairy farms in Kano State?
- Are animals shedding residues in milk more likely to be shedding Multi-Drug resistant *E.coli*?
- What proportion of *E.coli* isolates from the raw cow milk samples carry the *tet* genes?
- Does location affect occurrence of antibiotic residues?

CHAPTER TWO

LITERATURE REVIEW

2.1 The Bacterium *Escherichia coli*

Escherichia coli historically was first isolated from the faeces of a child in 1885 by Theodor Eschrich and have since remained the most studied bacterium. It is a common inhabitant of the gastrointestinal tract of animals and humans (Kaper *et al.*, 2004). There are two broad types of *E. coli*, the *E. coli* strains that are harmless commensals of the intestinal tract and others that are major pathogens of human and animals. While the pathogenic *E. coli* strain is divided into those causing disease inside the intestinal tract and others capable of infection at extra-intestinal sites (Sousa, 2006). *Escherichia coli* is found secondarily in soil and water due to faecal contamination. The bacterium can be cultured easily in the laboratory, the different pathogenic genotypes can be identified through virulence gene detection methods (Sousa, 2006). Coliform bacteria possess variation in their morphology including *E. coli*. The usual morphology observed in the stained preparation after culture on nutrient agar range from 2 to 4 microns in length and 0.4 to 0.7 microns in breadth (Hahn, 1996).

2.2 *Escherichia coli* Contamination of Milk

Escherichia coli frequently contaminate food and can serve as a good indicator of faecal pollution (Dilielo, 1982; Soomro *et al.*, 2002; Benkemoun *et al.*, 2004). The role of *E. coli* in milk and milk products as argued by Hahn (1996) as a possible cause of food-borne disease is insignificant if the organism remains ubiquitous, normal and unpathogenic. However, presence of *E. coli* in milk indicates the presence of enteropathogenic microorganisms, which are known to have public health implications,

as they are presumed to be responsible for severe diarrhoea and vomiting in infants and young children (Asmahan and Warda, 2011).

Escherichia coli is the most prevalent infecting organism in the family of gram-negative bacteria known as enterobacteriaceae (Eisenstein and Zaleznik, 2000). *E. coli* that are responsible for the numerous reports of contaminated foods and beverages are those that produce Shiga toxin, so called because the toxin is virtually identical to that produced by *Shigella dysenteria* type 1 (Griffin and Tauxe, 1991). The best-known and also most notorious *E. coli* bacteria that produce Shiga toxin is *E. coli* O157:H7 (Griffin and Tauxe,1991; Eisenstein and Zaleznik, 2000). Shigatoxin producing E. *coli* (STEC) caus es approximately 100,000 illnesses, 3,000 hospitalizations and 90 deaths annually in the United States (Mead *et al.*, 1999). Most reported STEC infections in the United States are caused by *E. coli* O157:H7, with an estimated 73,000 cases occurring each year (Mead *et al.*, 1999).

Escherichia coli is one of the main inhabitants of the intestinal tract of most mammalian species, including humans, cattle and birds. Shiga toxin-producing *E. coli* (STEC), also called verotoxinogenic *E. coli*, do not cause disease in animals but may cause watery diarrhoea, haemorrhagic colitis, and/or haemolytic uraemic syndrome in humans (Fairbrother and Nadeau, 2006). Zoonotic STEC include the O157:H7 strains and, with increasing frequency, certain non-O157 strains. The importance of non-O157 zoonotic strains is probably underestimated as they have been less well characterised and are more difficult to detect in samples than O157:H7 (Fairbrother and Nadeau, 2006).

Another large subset of STEC strains has been isolated from animals but has not, at the present time, been associated with disease in animals or humans. Cattle and other ruminants are the most important reservoir of zoonotic STEC, which are transmitted to humans through the ingestion of foods or water contaminated with animal faeces, or through direct contact with the infected animals or their environment (Fairbrother and Nadeau, 2006).

Pathogenic members of the coliform group as well as the Enterobacteriacae family are represented by genera such as *Salmonella* and *Shigella* and, are often found in the intestines of humans and animals (Le Minor, 1984; Rowe and Gross, 1984; Collins *et al*., 1995; Hayes *et al*., 2001). Most strains of *E. coli* are non-pathogenic (Stender *et al.*, 2001). However some strains differ from commensal in that they express virulence factors directly involved in pathogenesis thereby causing disease (Schroeder *et al.*, 2004). *E. coli* frequently contaminates food and is a good indicator of faecal pollution (Dilielo, 1982; Soomro *et al*., 2002; Benkemoun *et al.*, 2004). Presence of pathogenic *E. coli* in milk products indicates the presence of enteropathogenic microorganisms, which constitute a public health hazard.

2.3 Distribution And Diversity Of *Escherichia coli* In Dairy Farms

Recent increase in human illness due to pathogenic *E. coli* has raised considerable interest in the diversity and distribution of *E. coli* (EPA-OST, 2000). *Escherichia coli* form a complex microbial community in the gastrointestinal tracts of healthy mammals. Although some strains of *E. coli* are pathogenic, the degree of pathogenic potentials varies between strains and pathogenic forms (Thorpe *et al.*, 2002), and they represent a small proportion of the species.

Dairy cattle are known reservoirs of both pathogenic and non-pathogenic *E. coli*, but little is known about the dynamics of *E. coli* in dairy cows or within the dairy farm environment (Wells *et al.,* 1991; Whipp *et al.,* 1994). Dairy farms are complex environment (Son *et al.,* 2009) where milking cows are moved at least twice daily through common alleys to a milking parlor, and manure is typically moved daily to a storage unit and then spread on the fields at later date. A typical dairy cow can excrete 40 to 50 kg of faeces daily (Wilkerson *et al.,* 1997).

Because *E. coli* is ubiquitous in faeces, phenotypic and genotypic typing of the organism has been proposed as a means of tracking sources of faecal contamination (Welinder-Olsson *et al.,* 2002; Lahti *et al.,* 2003; Avery *et al.,* 2004; Arthur *et al.,* 2007; Son *et al.,* 2009). Some studies have demonstrated high genomic variability among commensal non-pathogenic *E. coli* strains from healthy pigs, cattle and human (Jarvis *et al.,* 2000; Duriez *et al.,* 2001; Leung *et al.,* 2004; Higgins *et al.,* 2007; Houser *et al.,* 2008).

2.4 Laboratory Diagnosis of *Escherichia coli*

Microscopic examination at X100 magnification of stool samples cultured on eosin methylene blue agar (EMB) and MacConkey agar shows clusters of Gram negative rods. On EMB they appear as black colonies with greenish-black metallic sheen while, on MacConkey agar, pink or deep red colonies are produced to confirm the lactose fermenter organism resulting to drop in pH, leading to darkening of the medium. Biochemical tests used for confirmation of *E. coli* include triple sugar iron test (TSI) (A/A/g+/H2S), IMViC; indole (red ring for positive), Methyl red (bright red for the

positive), vogues proskeur negative (no colour changes) and citrate negative (colour remains greenish).

For *E. coli* 0157:H7 in stool, ELISA test, direct immunoflorescent microscopy of filters and immune capture techniques using magnetic beads can be used in its diagnosis. All these are screening tools that precede rapid testing for the presence of *E. coli* without prior culturing of the stool (Heuvelink and Boer, 2000). More recent kits like microbact E and Microgene are also used for identification and isolation of *E. coli*.

2.4.1 Detection and enumeration of *Escherichia coli* in milk

Detection and enumeration of *E. coli* in milk can be assessed by various methods including plate counting, immuno-magnetic separation, flow cytometry and chromogenic-fluorogenic substrate technology (Venkateswaran *et al.*, 1996; de Boer, 1998; Seo *et al.*, 1998; Yu, 1998). However, the standard detection method for *E. coli* involves the use of substrates that detect *E. coli* and this can be time consuming (Yokoigawa *et al.*, 1999; Manafi, 2000; Daly *et al.*, 2002).

Polymerase chain reaction (PCR) has led to rapid and sensitive detection of *E. coli* from clinical samples and food types (Fratamico *et al.*, 2000; McKillip and Drake, 2000; Hsu and Tsen, 2001). However, many food types contain PCR inhibitors which co-purify with the target DNA (Gonzalez *et al.*, 1999), thereby requiring extensive sample preparation to remove, dilute or inactivate inhibitors prior to PCR amplifications (Fratamico *et al.*, 2000).

Nevertheless, in many cases, the low level of *E. coli* in food types requires a pre-enrichment step to overcome poor sensitivity, which increases the overall assay time by

up to 24hrs (Seo *et al.,* 1998; Scotter *et al.,* 2000). Thus detection of *E. coli* in milk using PCR may be useful (Yokoigawa *et al.,* 1999).

2.5 *Escherichia coli* as an Indicator Organism

Coliforms and *E. coli* are often generally used as marker organisms. Recovery and counting of *E. coli* is a reliable indicator of faecal contamination and indicates a possible presence of enteropathogenic and/or toxigenic microorganisms which constitute a public health hazard (Kaper *et al.,* 2004). In recent years, *E. coli* has become recognized as a serious food-borne pathogen and has been associated with numerous outbreaks of disease around the globe (Uyttendaele *et al.,* 1999; Scotter *et al.,* 2000).

One major inhabitants of the intestinal tract of mammalian species, including man and birds is *E. coli*. Most *E. coli* are harmless, but some are believed to be pathogenic, causing severe intestinal and extra intestinal diseases in human (Kaper *et al.,* 2004). *Escherichia coli* is a normal microflora of the intestinal tract of humans and warm-blooded animals. The organism is a known causative agent of diarrhoea and other food-borne related illnesses through the ingestion of contaminated foodstuffs (Le Minor, 1984; Rowe and Gross, 1984; Collins *et al.,* 1995; Hayes *et al.,* 2001). Most *E. coli* strains are non-pathogenic; however, some strains differ from commensal in that they express virulence factors directly involved in disease pathogenesis (Stender *et al.,* 2001; Schroeder *et al.,* 2004).

2.6 Use of Antibiotics in Animal Production

Veterinary drugs are pharmacologically and biologically active chemical agents especially designed for treatment and prevention of animal diseases (Navratilova, 2008). Veterinary drugs at present are extensively used in animal production, due to its gigantic growth and intensification (Botsoglou and Fletouris, 2001). The most important and most frequently used group of veterinary drugs is that of antimicrobial agents (Fischer *et al.,* 2003).

In lactating cows, antimicrobial agents are used mostly for the therapy of mastitis but also of other diseases (Navratilova, 2008). Long-acting antimicrobials are used commonly in dry-cow therapy (Honkanen-Buzalski and Shuren, 1999; Botsoglou and Fletouris, 2001). Antimicrobial agents administered to cows in the course of lactation can pass into milk in various levels. A frequent and prevailing source of drug residue in milk is the intramammary (intracisternal) administration of a specific antibiotic (Heeschen and Bluthgen, 1991).

Producers administer antibiotics to food animals to promote growth and to enhance feed efficiency (DeSchrijver *et al.,* 1990). Various sources provide data on such uses in animals (Arello, 1998). Therapeutic treatments are intended for animals that are diseased. In food animal production, individual animals may be treated or medicating feed or water (AVMA, 2001; Kabir *et al.,* 2004). In addition, subtherapeutic concentrations are commonly added to animal feed and/or drinking water as growth promoters, and have been a regular part of food animal production (Cromwell, 2001; McEwan and Ferdorka-Cray, 2002). Antibiotics can select for resistant bacteria in the gastrointestinal tract of animals, providing a potential reservoir for their dissemination

to animals, humans and the environment. The potential for long-term, cumulative inputs of antibiotics and their potential effects on acquisition and maintenance of antibiotic resistance mechanisms in bacteria, suggest a degree impact on the occurrence, persistence and mobility of resistance genes in natural environments (Andremont, 2003).

A number of reviews, reports and opinion papers have emerged to address the possible link between antibiotic use and the impact on antibiotic resistance development (Gustafson and Bowen, 1997; Khachatourians, 1998; USGAO, 1999; Isaacson and Torrence, 2002; Seveno *et al.,* 2002; Kummerer, 2004). These reviews have highlighted various issues related to antibiotic use in agriculture, often focusing on the link to emerging antibiotic-resistant bacteria, gene transfer mechanisms and consequent risks to human and animal health.

2.6.1 Antimicrobial feed additive

A broad range of antimicrobial compounds used against pathogenic microorganisms exists, and are used as feed additives for dairy cattle (Huber *et al.*, 1971a). Antibiotics incorporated into livestock feeds include the tetracyclines (chlortetracycline, oxytetracycline, tetracycline), aminoglycosides (streptomycin, dihydrostreptomycin, spiramycin, tylosin), synthetic antibiotics (chloramphenicol), polypeptide antibiotics (bacitracin, colistin) and ionophore antibiotics (lasalocid, monensin, salinomycin). Others include flavomycin and virginiamycin (WHO, 1969; Solomon, 1978).

The combined use of antibiotics results is a synergistic effect (Beason, 1969). According to Wallace (1970) and Visek (1978), antibacterial feed additives inhibit or suppress the growth of microorganisms by having an indirect metabolic effect on the

metabolism of digestive tract microflora. They also make certain nutrients available to the host and enhance the effectiveness of disease control measures (Dafwang *et al.*, 1987).

2.7 Concern on Drug Residues in Food

The indiscriminate use of antimicrobials drugs by livestock owners/farmers might result in deposition of residues and this has elicited some concern over residues in food of animal origin which occurs as a potential threat of direct toxicity in human. This may result in alteration of microflora, cause disease and the possible development of resistant strains which cause failure of antibiotic therapy in clinical situations (Nisha, 2008). Presently, international and national committees have evaluated data on the chemical, pharmacological, toxicological and microbial properties of veterinary drugs so as to assess the safety of ingested antimicrobial residues to human (Fink-Gremmels and Van Miert, 1994; Woodward, 1998). However, European Union and the United States guidelines for veterinary drug registration recommend that the microbiological hazards from antimicrobial residues must take into account the potentially harmful effects of drug residues on the human gut (JECFA, 1991a; USFDA, 1993; 1996; CVMP, 1995).

2.8 Effects of Antimicrobial Drug Residues

Antibiotic Residues in Food produce some adverse effects such as; Inhibiton of starter cultures, Transfer of antibiotic resistant bacteria to the human, Immunopathological effects,Autoimmunity,Carcinogenicity(Sulphamethazine,Oxytetracycline,Furazolidone), Mutagenicity,Nephropathy (Gentamicin), Hepatotoxicity, Reproductive disorders, Bone marrow toxicity (Chloramphenicol), Allergy (Penicillin).

2.8.1 Effects of antimicrobial drug residues on human health

The public health risk associated with antimicrobial residues depends on the quantity of the antimicrobial encountered or consumed (i.e. the exposure). The presence of antibiotic residues in foods of animal origin like milk is a potential health threat (Duran and Marshall, 2005) to humans. It is important to consider that most antibiotics used for treating infections are produced by environmental micro-organisms, meaning that the genes for antibiotic resistance must also have emerged in non-clinical/artificial habitats (Martínez 2008).

When consumed directly by humans as medicine, antibiotics may cause adverse side-effects, but these can generally be avoided through adhering to the recommended dose and duration of therapy. However, when antibiotics are unintentionally ingested as residues in food, the amount ingested cannot be quantified or monitored and may cause direct health concerns (IOM, 1989). Recognition of the risks associated with the direct and indirect effects on human health of both active and passive consumption of antibiotics has led to bans on the use of certain antibiotics in animal food production (particularly those antibiotics for which no safe residue levels can be determined) and to the establishment of maximum residue limits (MRLs) for those with known risks (FAO, 2002). When antibiotics are unintentionally ingested as residues in food, the amount ingested cannot be quantified or monitored and may lead to direct health concerns, such as aplastic anaemia, which is linked to chloramphenicol. These direct effects pose significant risks to human health (Alderman and Hastings, 1998).

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2.8.1.1 Antibacterial drug resistance

Microbes manifest resistance to antibacterial drugs through different mechanisms. Some bacteria are innately resistant to more than one class of antimicrobial drugs (Tenover, 2006). In such cases, all strains of that bacteria species are likely to develop resistance to all members of those antibacterial classes. Cases of acquired resistance are of greater concern, where bacteria populations initially susceptible become resistant to an antibacterial agent and proliferate and spread under the selective pressure of use of that drug (McEwan and Ferdorka-Cray, 2002).

Varied mechanisms of antimicrobial resistance are readily spread to several bacteria genera. First, the organism may acquire genes encoding enzymes such as β-lactamases that destroy the antibacterial agent before it can have an effect (McManus, 1997). Secondly, bacteria may acquire efflux pumps that extrude the antibacterial agent from the cell before it can reach its target site and exert its effect (Poole and Srikumar, 2001; Schweizer, 2003). Thirdly, bacteria may acquire several genes for a metabolic pathway which ultimately produces altered bacteria cell walls that no longer contain the binding site for the antimicrobial agent, or bacteria may acquire mutations that limit access of antimicrobial agents to the intracellular targets site via down-regulation of porin genes (Singh *et al.,* 2005; Maurelli, 2006). This may occur either through one of several genetic mechanisms such as transformation, conjugation or transduction (Bester and Essack, 2010).

Through genetic exchange mechanisms, many bacteria have become resistant to multiple classes of antibacterial agents, and these bacterial with resistance to more than one or three antibacterial drug classes (multiple drug resistance) have become a serious global concern (Tenover, 2006). Strains of bacteria carrying resistance conferring mutations are selected by antimicrobial use, which kills the susceptible strains but allows the newly-resistant strains to survive and grow. Acquired resistance that develops due to chromosomal mutation and selection is termed vertical evolution (Roh *et al.,* 2010). Bacterial also acquire resistance through the acquisition of new genetic materials from other resistant organisms. This is termed horizontal evolution, and may occur between strains of the same species or between different species or genera. The mechanism of genetic exchange includes conjugation, transduction and transformation (McManus, 1997; Bester and Essack, 2010). For each of these processes, transposons may facilitate the transfer and incorporation of the acquired resistance genes into the host's genome or into plasmids.

Pathways for antibiotic resistance transfer

Direct (primary) and indirect (secondary) pathways are linked to the problem of acquired antibiotic resistance. The primary pathways are mutations in the gene encoding resistance against the mechanisms of particular antibiotics (Dessen *et al.,* 2001; Catry *et al.,* 2003; Ghosh and LaPara, 2007). While secondary pathways are gaining of small fragments of DNA coding for resistance. These secondary pathways are further categorized into three: transformation, conjugation and transduction (Kelly *et al.,* 2009a).

Transformation pathway

Transformation is the process by which bacteria acquire and incorporate DNA segments from other bacteria that have released their DNA complement into the environment after cell lysis (Bester and Essack, 2010). Mutation and selection, together with the mechanisms of genetic exchange, enable many bacterial species to adapt quickly to the introduction of antimicrobial agents into their environment (Neu, 1992; McGowan, 2001).

Transformation pathway occurs when a dying pathogen releases its plasmids, or short fragments of its DNA into the environment, allowing a healthy pathogen or recipient to acquire the plasmid or DNA short fragment and use it for its own benefit (Bester and Essack, 2010) directly through the cell wall (Catry *et al.,* 2003). However, this form of genetic transformation has limitations; such an action is possible only if the recipient has the requisite genetic capacity to absorb 'loose' compatible DNA (usually in a plasmid form), this can only occur in limited bacteria pathogens (Maurelli, 2006; Kelly *et al.,* 2009b).

Conjugation pathway

During conjugation, a Gram negative bacterium transfers plasmid containing resistance genes to an adjacent bacterium, often via an elongated proteinaceous structure termed pilus, which joins the two organisms. While conjugation in Gram positive bacteria is usually initiated by production of sex pheromones by the mating pair, which facilitate the clumping of donor and recipient organisms, allowing the exchange of DNA (Catry *et al.,* 2003; Kelly *et al.,* 2009a; Roh *et al.,* 2010). The conjugation pathway allows transmissible plasmids and chromosomal DNA of very large sizes to be transferred from

cell to cell, either within or between species, mediating the transmitted genetic material through various specified enzyme activities (Catry *et al.*, 2003).

This pathway does not select DNA material simply from the environment, but has a direct cell-to-cell exchange of genetic material, where one cell is the donor of the genetic material and the other the new host or the recipient (Bester and Essack, 2010). This pathway uses a very specific, hair-like attachment on the surface of the bacterial cell, constructed mainly of oligomericpilin protein, which acts as a bridge pulling the two cells together (Catry *et al.*, 2003). Enzyme activities further initiates the transportation of a single DNA strand to the new host, where both cells synthesis duplicates, and both cells can act as donor cells (Catry *et al.*, 2003; Bester and Essack, 2010). Additional role players that use such pathways to assist the bacterial strain to obtain antimicrobial resistance and pathogenicity include plasmids, pathogenicity islands, transposons, integrons (Singh *et al.*, 2005; Kelly *et al.*, 2009a) and insertion sequences (Roh *et al.*, 2010).

Conjugation plasmids that have the ability to replicate independently in the recipient or newly acquired host have greater opportunity to spread through a bacterial flora community than those without conjugative abilities (Maurelli, 2006). It is unclear whether the transfer of genetic elements through conjugation occurs voluntarily or equally in cells or whether cell 'hijacking' occurs for the survival of a more aggressive genetic element (Bester and Essack, 2010).

Transduction pathway

In transduction, resistance genes are transferred from one bacteria to another via bacteriophage (bacterial viruses). Transduction is the virus-like injection of genetic

material into a host cell after attachment. Current knowledge postulates that transduction is a feature mainly of bacteriophage (Maurelli, 2006; Bester and Essack, 2010).

2.8.1.2 Antibiotic selective pressure

Selective pressure imposed by the use of antimicrobials in both human and veterinary medicine promotes the spread of multiple antimicrobial resistances resulting in the growing problems of infections that are difficult to treat (Threlfall, 2002; Carattoli, 2003). Microbial resistances to some β-lactams, tetracyclines, chloramphenicol or trimethoprim are reported with increasing proportion (Velonakis *et al.,* 2001). Selective pressure on antimicrobials results in adverse effect of antimicrobial resistance, and this has been recognized as treatment failure. The disease caused by the pathogen may be worsened significantly due to the incurred resistance (Clarence and Nosakhare, 2008).

Concern on antimicrobial resistance in animal and human health

Several reasons exist why antimicrobial resistance is a concern not only to physicians and veterinarians alone but also the public (Barbosa and Levy, 2000; Courvalin, 2005). Foremost, resistance bacteria such as *E. coli,* Enterococci and Staphylococci are commonly associated in health care institutions and farms (Chambers, 2001 and Karlowsky *et al.,* 2003). Inadequate empirical antibacterial therapy (the initial use of an antibacterial drug to which the causative agent is not susceptible), has been associated with increased mortality rates in patients with bloodstream infections due to resistant bacteria organisms (Ibrahim *et al.,* 2000; Kang *et al.,* 2005). Resistant bacteria may also spread and become broader infection-control problem (Pitout *et al.,* 2004; Woodford *et al.,* 2004). The spread of resistant microorganisms poses additional problems for

infection control globally and places added burden on healthcare costs (McGowan, 2001).

Antimicrobial resistance is also a concern for animal health (McEwan and Ferdorka-Cray, 2002). Resistance among animal pathogens reduces the effectiveness of some drugs (Wray and Gnanou, 2000). This effect could potentially affect public health if drug use in food animals increases to compensate for this drop in effectiveness or if alternative drugs that are crucial to human health are used to treat animals (NAS, 1999; Rosssolina and Thaller, 2010). Antimicrobial resistance has been reported in a wide variety of animal pathogens e.g., *E. coli* of calves, pigs and poultry (Martel and Coudert, 1993; Lee and Maurer 2000). Generally, there is high variability among animal pathogens in different geographical areas (Dargatz *et al.,* 2000; Wells *et al.,* 2001). Furthermore, some isolates of pathogens such as *E. coli* are resistant to multiple antimicrobials (McEwan and Ferdorka-Cray, 2002).

2.8.1.3 Toxic effects

Residues occur as free from parent drug or its metabolites, conjugated or covalently bound to tissue components. Certain toxicities are mediated by metabolites of otherwise inert substances that covalently bind to tissue macromolecules like DNA and enzymes. Thus, residues may have a direct toxic effect (Gillete, 1974; WHO, 1988). Toxic effects associated with drug residues are confined to allergic reactions in man. Adverse reactions to tetracyclines following consumption of food contaminated with tetracycline residues have been reported (Anthony, 1977). Similarly, photo-allergy and phototoxicity are known to occur in individuals undergoing therapy with chlortetracycline.

Other toxic effects observed in human and also in experimental studies in bacteria, dogs, mice, rabbit and rats include adverse drug reactions following therapy, carcinogenicity, mutagenicity and teratogenicity (WHO, 1990). Tetracyclines have been reported to cause a genetically determined disorder characterized by defects in renal tubular function due to ingestion of degraded tetracyclines (fanconi syndrome). Similarly, tetracyclines are reported to sequester into bones and cause a reversible cessation of bone growth and discoloration (Gross, 1963; WHO, 1990).

2.8.1.4 Environmental contamination

Resistant organisms from both animals and human may result in the pollution of the environment. Antibiotic resistant bacteria from animals to human present a grave concern. Reports demonstrating the close relationship between incidence of antibiotic resistant strains isolated from animals and humans exists (Smith, 1974). Certain factors such as age, use of antacids, previous exposure to antibiotics have been suggested to favor disease occurrence with resistant bacteria (Riley *et al.*, 1984).

2.9 Antimicrobial Drug Residues in Milk

The presence of antimicrobial residues of much higher levels in milk may constitute a variety of public health hazards including toxicological, microbiological, immunological, and pharmacological hazards (Heeschen, 1993). Most important health risks associated with antimicrobial residues in milk include possible impact on the emergence of antimicrobial resistance for antimicrobial administered in human therapy, disorders in the intestine flora, and possible occurrence of allergic symptoms (Honkanen-Buzalski and Reybroeck, 1997; Roberts, 1997; Cerniglia and Kotarski, 2005).

The presence of a drug residue may render a product adulterated and thus constitute a violation of the law (Guest and Paige, 1991). An overview of field surveys in the United States revealed drugs most commonly involved in residues included streptomycin (20.2 %), Penicillin (31.1 %), and oxytetracycline (9.1 %) (Van Dresser and Wilke, 1989).

According to Naravtilova *et al.* (2009), about 1 % of products of animal origin in the United States and Europe contain antibiotic residues in very low concentration. Where as, the cause for the incidence of the antibiotic residues in milk is 92 % due to their administration in mastitis therapy. However, in most countries, the most frequently detected antibiotics in milk are β-lactams and tetracyclines (Schmidt and Rodrick, 2003). In a study, Navritilova *et al.* (2009) reported the presence of tetracycline residues in raw milk produced in Czech Republic, and they attributed the presence to its frequent use in lactating cows. It is assumed that further processing of milk can lower the concentration of tetracyclines (Moats, 1999), further still, it is presume that during heat treatment of milk, there is only partial reduction in the concentrations of tetracycline residues. For instance, temperature of 60^{o}C over a period of 30 minutes, caused reduction by 16 % and 23 % of chlortetracycline and oxytetracycline contents respectively, while a temperature of 72^{o}C over the same time period caused a reduction by 27 % and 35 % in chlortetracycline and oxytetracycline contents, respectively (Moats, 1999). The residual presence of tetracyclines and other antimicrobials in milk presents technological difficulties in the dairy industry (Heeschen and Bluthgen, 1991). Hence, the widespread use of antimicrobials in dairy cattle management may result in the presence of antibiotic residues in milk (Navratilova *et al.,* 2009).

2.9.1 Determinants of antimicrobial residues in dairy herds

Factors influencing the occurrence of antibacterial residues in the dairy industry have been investigated. These factors favoring the occurrence of these drug residues are diverse and often complicate each other. They include; age of the animal, indiscriminate drug use in dairy farms, management practice, status of the animal and pharmacokinetic parameters (Codex,2000).

2.9.1.1 Pharmacokinetic parameters of the drug

Varying pharmacokinetic indices influences the persistence of administered antibiotics, and hence, the persistence of residues in milk of dairy cows. Oxytetracycline is one drug amongst others that are found as residues in milk from dairy cattle (Seymour *et al.,* 1988; Guest and Paige, 1991).

Nature of the drug

The form of antibacterial, such as those associated with slow release preparation have been incriminated in causing residue. Similarly, the chemical nature of the drug and the use of boluses can cause the presence of residues (Archibault, 1982).

Dosage and dose rates

Extra-label drug use exceeding recommended dose administration has often accounted for residues in milk and other tissues. The higher the dose, the longer its persistence in the milk. Likewise, increasing the dose of a drug extends the withdrawal time by one biological half-life of the drug (McEwen *et al.,* 1991; Riviere, 1991).

Neglect of withdrawal period

The failure to observe drug withdrawal time has been established as a leading cause of drug residues in milk (Johnson *et al.*, 1977; Bevill, 1984; Oliver *et al.*, 1984; Guest and Paige, 1991; Kabir, 1998).

Route of drug administration

Certain routes of drug administration and poor bioavailability have been associated with risk of drug residues in milk. Injectable drugs are more likely to result in antibacterial residues. Intravenous and intramuscular routes of administration showed greater potential to cause oxytetracycline residues in milk than other routes (Guest and Paige, 1991; Van Dresser and Wilcke, 1991; Anderson *et al.*, 1995).

Sequestration and protein binding

Persistence of drugs in plasma is responsible for their covalent bindings to macromolecules, proteins and other biological components, which depends on the ability of the drugs to get into systemic circulation and its subsequent sequestration into tissues (Van Dresser and Wilcke, 1991). Drugs administered systematically, in plasma may sequester into milk and deposit appreciable residues. Drugs which consist of weak bases have a greater potential to cross the blood mammary epithelium barrier and concentrate in milk (Baggot, 1977; Heeschen and Bluthgen, 1982). Intrauterine infusion of some antibiotics caused detectable levels of residue in milk. Besides, antibiotics have been reported to have transferred from treated to untreated quarters in dairy cows (Black and Claxton, 1982).

Handling of milk after harvest

Treatment of milk could alter the content or eliminates the presence of residues. Ultra heat treatment (150^{o}C for 7.9 seconds) of milk containing antibiotic inactivated tetracycline (Yingprayoon, 1989).

2.10 Detection of Veterinary Drug Residues

In developed countries, only registered veterinary drugs are allowed to be used in food-producing animals, protection periods are prescribed during which the quantity of residues in foodstuffs of animal origin (meat, milk, egg) should be reduced to a level not threatening to consumers health. Countries world-wide rely on national regulatory agencies and international committees in evaluating the safety of all drugs used for food animals for potential human health risk as an integral part of the drug registration process (Navratilova, 2008).

The Codex Alimentarius and Joint FAO/WHO have developed standards on drug residues in food since 1985. These standards are based upon scientific assessments performed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) determining the Acceptable Daily Intakes (ADIs) and giving recommendations for Maximum Residue Limits (MRLs) (Heeschen, 1993; Herman, 1995; Honkanen-Buzalski and Reybroeck, 1997). Consequently, the approval of an antimicrobial drug for use as treatment in food animals should require the fixation of ADI, development of a suitable detection method and fixation of the withholding period on the basis of the residues (ADI/MRL) detected (Heeschen, 1993).

2.10.1 No observable effect levels

No observable effect levels (NOEL) is the maximum concentration of a drug in a feed that produce no observable pathophysiologic change in the laboratory animal expressed as drug per unit body weight. These on weight basis are extrapolated to human (WHO, 1990).

2.10.2 Acceptable daily intake

International and national committees define acceptable daily intake (ADI) of a veterinary drug as "an established amount which is safe in humans even if humans consume that amount every day" (Cerniglla and Kotarski, 1999). Acceptable daily intake (ADI) established for humans are derived from NOEL. The recommendation of ADI in humans uses a fraction of the levels in animals in its computation (a safety factor), additionally, a target tissue, a marker residue and an analytical method to quantitate the residue (Fitzpatrick *et al.*, 1995).

2.10.3 Maximum residue limit

Maximum Residue Limits (MRLs) are established based on food consumption values and observed drug residue concentrations that ensure that ADI are not exceeded. Once MRL is established for food from animal origin, a drug withdrawal time is determined that is specific for the animal species, dose and route of administration (CVM, 1994; CVMP, 1997). However established MRL and withdrawal time do not assure "zero risk" of residue occurring in food. In addition, the use of conservative measures in establishing the MRLs withdrawal times decreases the likelihood that consumers will ingest residues at the established ADI (Cerniglia and Kotarski, 1999).

Based on toxicological residue assessment, MRLs levels are set for pharmacologically active chemical agents of veterinary drugs occurring in food animals. To prevent any harmful effects on consumers, Food and Agricultural Organization and World Health Organization have established the MRLs for veterinary drugs. The MRL set for tetracycline, oxytetracycline, in raw milk is set to 0.1 mg/kg (100 ng/g) (Navratilova *et al.*, 2009). Maximum residue limits have been set under Council Registration (EEC) No. 2377/90 (as amended) ranging from 100 to 600 µg/kg dependent on the species and tissue type (Stead *et al.,* 2007). The current MRL for tetracycline residues in muscle tissue of food producing animals is 100 µg/kg (VMD, 2006). All veterinary drugs specified for food animals must be toxicologically assessed and categorized into Annexes I-V depending on the MRL type. Maximum residue limits present the intentionally acknowledge limits which specify maximum quantity of the drug residues that may be found in food of animal origin (Navratilova, 2008). The dairy industry must introduce measures ensuring that raw milk will not be marketed if it contains the residues of antibiotics in quantities exceeding levels for veterinary drugs, or if the overall content of all antibiotic residues exceeds the MRLs.

Currently, a method exists which can detect drug residues at the level of established MRL values. In order to provide for the high technologically quality of raw milk and, at the same time, the safety of the milk and milk products for the general public, there must be an integrated system of checking veterinary drugs in milk and milk products. This system should include various methods for the antibiotic detection and specifies the responsibility for the health safety of milk and milk products in the technological process of producing and processing milk (Honkanen-Buzalski and Reybroeck, 1997; Honkanen-Buzalski and Suhren, 1999).

2.10.4 Methods of determining antimicrobial residues in milk

There are three basic methods of determining antimicrobial residues; microbial methods, physicochemical methods and immunoassays. Mitchell *et al.* (1998) argue that microbial inhibitor test was the first test developed to screen and establish antimicrobial residues in milk. They further posit that methods for screening residues in milk should be relatively cheap, simple to carry out, and capable of detecting a wide variety of antimicrobial agents. Microbial inhibitor screening method best suit these requirements, however, a major limitation of this test is the long incubation period associated with it (Navratilova, 2008).

Several rapid assays with short incubation period for antibiotic detection in milk have been developed. They are simple, sensitive and specific (Mitchell *et al.,* 1998). Some of these rapid assays include Premitest, Penzyme test (1980s), Charm II test (1988), LacTec test (1991), SNAP test (1994), Beta Star test and Charm Safe Level test. The International Dairy Federation (IDF) have described methods that can be used for the detection, confirmation and establishment of antimicrobial residues in milk and milk products (Heeschen, 1993). Hence, for the general monitoring of antimicrobial agents in milk, common use is established of microbial inhibition, physico-chemical methods and rapid specific tests (Navratilova, 2008).

2.10.4.1 Microbiological inhibition methods

Microbiological methods of determining antimicrobial drug residues have been shown to be suitable for screening purposes to estimate the presence and load of antimicrobial residues in milk (Moreno *et al.,* 1980; Singer and Katz, 1985).

Microbial inhibition tests

Microbial inhibition tests (MIT) is used in screening for residual antimicrobial agents in milk. The disc assay method has been used to detect antimicrobial substances in milk (Silverman and Kosikowski, 1952). The MIT modifications currently in use include: calf antibiotic sulfa test (CAST) using *Bacillus megaterium* or Mueller Hinton agar at 44^{o}C, the use of swab sticks-swab tests on premises (STOP), and the use of filter paper discs as sample applicator (Kosrud *et al.*, 1987; Kosrud and Macneil, 1988). The test organism used in the disc assay for screening milk is *Bacillus subtilis* ATCC 6633 (Silverman and Kosikowski, 1952; Macaulay and Packard, 1981). The sensitivity of this organism has made it the choice for use in different methods of screening milk (Huhtanen *et al.*, 1977; Kelley, 1982).

Microbial growth inhibition methods make use of a standard culture of the tested microorganism in a liquid or solid medium, such as *Escherichia coli*, *Bacillus megaterium*, *Sarcina lutea* and *Geobacillus stearothermophilus* (Heeschen, 1993). The analyzed milk sample is applied on the agar surface either directly or with a paper disc (disc assay plate method). If the sample contains inhibitor agent, reduction or total inhibition of the growth of the tested microorganism occurs. The presence of inhibitor agents in the milk sample is indicated by the formation of a clear zone of inhibition around the disc or a change in the medium color (Hui, 1993; Botsoglou and Fletouris, 2001).

These methods vary with the type of the testing organism, indicator incubation period and temperature spectrum and, detection levels of the agent analyzed. Commercially produced microbial inhibitor tests are applied simultaneously with selective rapid tests for milk screening in primary production, in dairy industry, and in accredited

laboratories (Suhren, 1995). According to Mitchell *et al*. (1998), these tests have both advantages as well as limitations. The advantages include a wide spectrum of detection, simple to carry out, cost effective, and ability to screen large number of samples. However, some of the draw-backs that limit their use include; they do not assure specific antibiotic identification, they have limited detection levels for a series of antibiotics, and they are only qualitative and require a long incubation period.

Several studies by some authors (Carlsson *et al.,* 1989; Andrew, 2001; Kang and Kondo, 2001; Kang *et al.,* 2005) have reported that natural antimicrobial agents when present in milk in higher concentrations can bring about false-positive results. Commercially produced microbial inhibition tests are delivered in ampoules (monotests) or in the form of microplates with a high number of testing cells (Althaus *et al.,* 2003). When carrying out this test, it is essential to observe good laboratory practice (protecting the test from contamination), check the pH value of the sample, observe carefully the correct temperature and incubation period as instructed by the manufacturers and testing a positive as well as a negative control alongside with the samples. Commonly used microbial screening methods include Charm cowside test, Charm farm test, Copan milk test, Eclipse test, premitest and others (Navratilova, 2008).

Premitest

This is a broadspectrum microbial screening test for detection of antimicrobial and sulphonamide residues in foods. It is a qualitative test that can detect residues at or above the MRL set by W.H.O in foods.

Principles of the test;

Premitest is a broad spectrum microbial screening test especially developed for the detection of antimicrobial substances, such as antibiotic and sulphonamide residues, in fresh meat, milk, fish, and eggs at or below W.H.O maximum residue level (MRL). The principle of the test is based on the inhibition of the growth of *Bacillus stearothermophilus*, a micro-organism very sensitive to many antibiotics and sulphonamide residues. A standardized number of spores are imbedded in an agar medium with selected nutrients. When milk sample is added to the Premitest and heated at 64°C, the spores will germinate. The germinated spores will multiply and form an acid when no inhibitory substances are present. This will be visible by a color change from purple to yellow of the indicator in the ampoule. When antimicrobial residues are present in sufficient amount, no growth will occur and the color will remain purple.

Premitest can be modified for the detection of tetracycline antibiotics using a calcium containing buffer. The ability of the tetracycline compounds to chelate polyvalent metal cations is a well established property of this class. There are many extraction, clean up and analysis methods for tetracyclines reported in scientific literature based on transition metal ion chelation columns, e.g chelating sepharose fast flow columns (Carson 1993).

Calcium forms a 2:1 metal ion to ligand complex. Formation of the calcium complex involves the addition of one metal ion to the C-10, C-11 site with subsequent addition of a second metal ion at the C12 and C-1 site (Newman 1976). Using a calcium and sodium containing buffer, metal ion chelation was achieved (a mechanism for distrupting the antimicrobial activity of tetracyclines) thus selectively reversing the

primary Premi Test positive response into a secondary negative response. It was postulated that on formation of the metal ion chelation complex, tetracyclines can no longer permeate the bacterial cell membrane via binding to the active transport mechanism, the tet® efflux pump (Chopra and Roberts 2001).

2.10.4.2 Physico-chemical methods

Colorimetric Determination

Colorimetric determination rely on the production of chemical products of the drug that could be detected under ultraviolet. This method can recover ten microbial (sulfonamide) within range of 01-100 % at 100 ppb level in milk (Tishler, 1968).

Enzyme colorimetric assays

Bacteria excrete intracellular enzymes while in growth, which are believed to be the soluble forms of membrane-bound transpeptidases involved in the synthesis of their cell wall. These enzymes can act as carboxypeptidases or transpeptidases (Leyh-Bouille *et al.,* 1970). Natural substrates for these enzymes react with β-lactam structures. β-lactam antibiotics create a covalent bond with carboxypeptidases giving rise to a very stable complex which inhibit the enzyme activity (Massova and Mobashery, 1998).

High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) is a quantitative test that yields high performance and high speed compared with traditional column chromatography because of the forcibly pumped mobile phase. It is used to separate the components in a mixture, to identify each component and to quantify each component (Hussein and Khalil 2013). It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample

interacts slightly differently with the adsorbent material causing different flow rates for the different components and leading to the separation of the components as they flow out the column (Hussein and Khalil 2013).

Thin layer chromatography

Thin layer chromatography (TLC) bioautography method has been employed for general identification of antibiotic residues (Neidert *et al.*, 1987; Salibury *et al.*, 1989). Blakely *et al.* (1969) developed a paper chromatography for identification of the three common tetracycline in the tissues of fish using nitromethane, chloroform and pyridine mixtures. Bossuyt *et al.* (1976) reported the TLC procedure for determining the three common tetracyclines (tetracycline, oxytetracycline and chlortetracycline) in milk using Kieselguhr as stationary. A minimum detectable concentration of 0.3 µg/ml of milk has been reported with reference values of 0.356, 0.20 and 0.60 for tetracycline, oxytetracycline and chlortetracycline, respectively. These reference values were obtained by running plates in methanol-chloroform (9:1). This method rely on the use of bioauthography with *Bacillus cereus* var mycoides and Staphylococcus (Aszalos, *et al.*, 1968; Bossuyt *et al*., 1976). Chemical detection with 3 % ferric chloride in methanol gives a light spot for tetracycline (Betina, 1964).

Receptor binding assays

This is an alternative method for establishing the presence of β -lactam group of antibiotics. Beta-lactam specific receptor proteins or penicillin-binding proteins (PBP) are used successfully in some methods and commercially produced tests (such as Penzyme test, Beta Star test, SNAP test and others) for establishing β-lactam antibiotic residues (Gustavsson, 2003).

Penzyme tests

Penzyme test is a qualitative enzymatic colorimetric method for a rapid determination of β-lactam antibiotics in milk. The test principle is based on establishing the level of inaction of the carboxypeptidase enzyme by β-lactam antibiotics. These residues binds specifically with the enzyme and inactivate it, thus interfering with the bacterial cell wall formation. The degree of enzyme inactivation depends on the amount of antibiotics present in the sample (Cullor, 1993). The end products of the substrate and enzyme reaction are measured using redox color indicator and the comparison of the final color with the color chart provided with the kit (Mitchell *et al.*, 1998). Hydrogen peroxide is used to oxidise the organic redox indicator that will change into a pink-orange color compound, indicating a negative result. If the color is peach, the sample is close to the detection limit. If a yellow or yellow-orange color is observed (test-positive outcome), the sample is suspected of containing an antibiotic residue (Gustavsson and Sternesjo, 2004).

SNAP residue tests

This is an enzyme-linked receptor binding assay in which β-lactams are captured by a binding protein on a solid support adsorbent matrix housed in molded plastic unit. The SNAP residue test consists of SNAP device, pipette and sample tube. This test utilizes a β-lactam receptor protein conjugated to an enzyme. The assay procedure includes three simple steps with a total assay time of about 10 minutes for a sample (Bell *et al.*, 1995; Neaves, 1995; Gustavsson, 2003).

Beta star residue test

The test involves a specific β-lactam receptor linked to gold particles. It is a dipstick test that detects penicillin and cephalosporins. The receptor will react with the free β-

lactams contained in a milk sample during incubation. Dipstick is added after incubation, the mixture is transferred to a strip of immuno-chromatography paper where it migrates towards the test field (Gustavsson, 2003). The receptor protein will be captured by a biomolecule immobilized at the test field of the chromatography paper in milk samples free of β-lactam residues. Since the receptor protein is linked to gold particle, the captured protein-gold complex will appear as a pink-colored band. The color intensity of the test band is compared with that of the reference band. If the color intensity of the test band is weaker than that of the reference band, the sample is classified as positive (Gustavsson and Sternesjo, 2004).

Microbial receptor assays

CHARM I and II tests are qualitative microbial receptor assays. CHARM I test developed for β-lactams in milk was the first rapid test recognized by the Association of Official Analytical Chemists (AOAC) with a test time of 15 minutes (Navratilova, 2008). The CHARM I test, in 1984-1985 was further developed to a test for antibiotics including tetracyclines, sulfonamides, aminoglycosides, chloramphenicol, novobiocin and macrolides. Thus, the extended version of CHARM I is called CHARM II (Botsoglou and Fletouris, 2001). In tetracycline and chloramphenicol test kits, antibody coating is used. The test employs ^{14}C or ^{3}H-radiolabeled antibacterials (tracer reagent) to compete for the binding sites. The procedure is relatively fast and simple. During incubation, any antibiotic present in the milk will bind to its specific natural receptor site on the bacterial cell. The rate of binding is measured with a scintillation counter and compared to the positive and negative controls. The higher the amount of the antibiotic present in the sample, the lower the counts detected by the equipment (Cullor, 1993; Hui, 1993; Mitchell *et al.,* 1998).

2.10.4.3 Immunoassays

Immunochemical methods are based on the reaction of an antigen with an antibody, a reaction of antigenic determinants with the antibody linking site. Chemically, antigens are polymers-proteins, polypeptides, polysaccharides, or nucleoproteins. They incite specific immune response and also react specifically with the products of this response which are antibodies and immunocomplementary cells. Antibodies are proteins, specifically immunoglobulin's, formed by the host animal in response to the invasion by antigens (Roeder and Roeder, 2000; Stepaniak *et al.*, 2003).

Nonisotopic immunoassays such as enzyme-linked immunosorbent assay (ELISA), fluorescence polarisation immunoassay (FPIA), particle concentration immunoassay (PCIA), particle concentration fluorescence immunoassay (PCFIA) and monoclonal-based immunoassays, may likely play increasing role in antibiotics screening immunoassay determination (Roedre and Roedre, 2000). However, enzyme immunoanalysis (EIA) is the most frequently used immunochemical method for rapid diagnostics of veterinary drug residues. As an enzyme label, horse radish peroxidase, alkaline phosphatase, glucose oxidase, pyruvate dehydrogenase and recombinant β-galactosidase are used. These enzymes catalyse the reactions that cause the substrate degradation and form colored products that can be read spectrophotometrically or visually (Botsoglou and Fletouris, 2001). In addition, most immunoasssays are performed in the laboratory and takes longer time before outcome of the test is known.

However, the need for onsite screening using fast and easy to perform tests is increasing. A number of immunochemical test are commercially available in a kit format for many drugs. More so, a promising approach in the rapid detection of antimicrobial residue is the use of lateral flow devices or dip stick format (Van

Herwijnen, 2006). A lateral flow test consists of the following basic components: sample filter, conjugate pad, membrane test line, and control line. The sample is applied to the sample filter housed inside the device casing. The sample runs through the sample filter and conjugate pad. This conjugate pad contains a labeled antibody, specific for analysis under test. Antibiotics, if present will form a complex with the conjugate and migrate further slowly to the membrane in the test zone. The test zone contains an immobilized antibody specific for the analysis, but preferably not competing with the conjugate antibody for the same or adjacent epitopes. The test line according to Van Henwijnen (2006) will capture the migrating analyse-conjugate complex. The intensity of the test line correlates well with the amount of analyte in the sample. Immunoassay can be sensitive, class specific, accurate, and provide a means for a rapid screening of samples for antibiotics (Cullor *et al.,* 1993; Gustavsson and Sternesjo, 2004).

Enzyme immunoassay

Enzyme immunoassay (EIA) offers quickness, accuracy, reproducibility and sensitivity. This test have been found to be very rapid, convenient and inexpensive, but lack specificity and sensitivity (Bane *et al.,* 1989). Improved EIA with procedures have been elucidated and found to be suitable for screening purposes. Enzyme linked immunosorbent assay (ELISA) for drug residue is either competitive direct or indirect ELISA using enzymes labeled antibody or enzyme labeled drug (Haagsma and Van de Water, 1992).

Several commercial kits are presently available for antibiotic residue detection in urine. The assay may be quantitative or qualitative. Quantitative assays involve a spectrum of color reaction, the intensity of which depends on the concentration of the drug in the milk sample (Golden, 1991; Bushway and Fan, 1995). There are reports (Rohner *et al.,*

1985; Dixon-Holland, 1992) of determination of tetracycline, chlortetracycline and oxytetracycline with immunoassay procedure in milk and other tissues.

Radioimmunoassay

Radioimmunoassay (RIA) is used with antibiotics of high affinity, the ligands are labeled with ^{14}C, ^{3}H or ^{175}I (Steiner and Harris, 1992). The RIA has been used for trace analysis of drug residues in milk with a sensitivity of 1 ppb (Arnold and Somogyi, 1985). The precision of the test depends on the type of the tissue and concentration of the drug. A radio-receptor assay uses bacteria like *Bacillus stearothermophilus* bearing receptor for different antibiotics instead of an antibody to detect the presence of drugs (Bishop and White, 1984).

2.11 Surveillance and Monitoring of Veterinary Drug Residues in Milk

The general surveillance of veterinary drug residues is undertaken by the use of microbiological inhibition methods, which allow their detection (qualitative determination) and/or semi quantitative determination, and by using specific rapid testing. This is a form of screening test (Mitchell *et al.*, 1998; Botsoglou and Fletouris, 2001). Physicochemical methods are used primarily for the isolation, separation, quantification and confirmation of the presence of detrimental residues in milk samples. HPLC and liquid chromatography has become the most widely used separation techniques for determining tetracycline antibiotics in milk and other edible animal products (Schenck and Callery, 1998; Oka *et al.*, 2000).

The reasons for monitoring antimicrobial residues in foodstuffs and food of animal origin include the ethical ones (preventing undesired exposition of health consumers to therapeutical doses of drugs in milk), hygienic (protection against possible harmful

effects of the residues on the consumers health), technological (preventing the disruption of the fermentation processes) and ecological (Mayra-Makinen, 1995; Honkanen-Buzalski and Reybroeck, 1997).

2.11.1 Regulations of antimicrobial residues in milk

Individual countries have developed regulatory agencies due to public health considerations to curb the increasing threat of antimicrobial residues in foods of animal origin (Gracey, 1986). The Swann committee (Swan, 1969) was established by the United Kingdom to evaluate the dangers posed by the use of antibiotics in animal husbandry. In their recommendation, the committee encouraged that only drugs with little or no possibility of developing resistance in bacteria and those with no use in human medicine should be allowed for use as feed additives in livestock production (Brander and Pugh, 1977).

The broadest and most comprehensive regulatory infrastructure is found in the United States. The Center for Veterinary Medicine (CVM) of the Federal Drug Administration (FDA) and the Food Safety Inspection Service (FSIS) of the United States Department of Agriculture (USDA) are two separate agencies that form the basis for major regulations in antimicrobial use in food animals, and thus protect the public health (Benson, 1990; Guest and Paige, 1991).

There are outline strategies towards residue prevention in foods animal. Antibiotics added to feed should be subjected to regulation, thus medicated feeds should be given under veterinary prescription. Further still, substances with androgenic, oestrogenic, or gestagenic action should only be administered by a qualified veterinarian (Council,

1981). Antibiotics that do not increase resistance in bacteria, and those not use in human medicine are allowed and preferred, respectively. This has led to important changes in the perceptions and priorities of regulatory agencies with regard to antimicrobial usage, particularly the use of antimicrobials as growth promoters and prophylactic agents (Call *et al.*, 2008).

2.11.2 Regulations of drug residues in Nigeria

The Food and Drugs Decree (1974) and the draft Veterinary Public Health (Meat Hygiene) Decree (1992) form the basis for the use of veterinary drugs and regulation of veterinary drug residues in food of animal origin in Nigeria (Kabir, 1998; Kabir *et al.*, 2004). The Food and Drug Decree (1974) described drug as any substance or mixture of substances manufactured, sold, advertised for use in diagnosis, treatment, mitigation, or prevention of any disease, disorder, abnormal physical state or symptoms thereof in animals or humans. This description includes all the drugs evaluated by the Food and Agricultural Organization/World Health Organization (FAO/WHO) expert committee (WHO, 1994). Existing regulations under this decree provides for the monitoring and surveillance, through inspection, sampling, analysis, prohibition from sale and setting tolerances for various substances in foods (Kabir *et al.*, 2004).

The National Agency for Food and Drug Administration and Control (NAFDAC) have taken over the regulatory roles and powers of the Food and Drug Decree (1974). However, there are presently no specific regulations for residues of veterinary drugs in animal products (Kabir, 1998). It is imperative to always ensure a strict limitation on marketing of milk following a course of medicated feeding. There is often no legislative requirement, but even so milk producers are under an important moral obligation not to milk their cows for at least four weeks after treatment to allow antibiotic to clear from

the milk of the cow; otherwise such milk may be responsible for induction of antibiotic resistance in the pathogenic flora of the individual consuming them.

2.12 Tetracyclines

Tetracyclines rank among antimicrobial substances most frequently used in animal food production (Schmidt and Rodrick, 2003). Tetracyclines are broad-spectrum substances, with a wide range of activity against Gram-positive and Gram-negative bacteria, chlamydia, mycoplasma, protozoan parasites and rickettsiae (Chopra *et al.*, 1992; Roberts, 1996; Sundin, 2003). Tetracyclines according to Roberts (1996) were the first major group of antibiotics ascribed the term "broad-spectrum". Hence, they have been used extensively in the therapy of animal and human infections, as well as for prophylactic purposes in animals and plants and for growth promotion in food animals (IOM, 1998). Tetracyclines are continued to be used for treatment in a variety of intracellular bacteria and protozoan infections, as well as for non-infectious conditions (Chopra and Robert, 2001; Roberts, 2003).

In cattle, tetracyclines are used as therapeutic agents against respiratory, urinary and local infections (Sundin, 2003). A specific indication for administering tetracyclines in cattle is infectious mastitis. A frequent and pervading source of milk contamination is intramammary (intracisternal) administration of the drug. Other milk contamination paths are percutaneous, intrauterine, subcutaneous, intramuscular and intravenous administration (Heeschen and Bluthgen, 1991). Milk tetracycline contents reach 50- 60 % concentrations of those in the blood plasma (Botsoglou and Fletouris, 2001). The overall action of tetracycline is bacteriostatic, while the main goal of the antibacterial action of the drug is protein synthesis inhibition (Navratilova *et al.*, 2009). Tetracycline

binds to the bacterial 30S ribosomal subunit and prevents attachment of amino acyltRNA to the ribosomal receptor site (Chopra *et al.*, 1992; Roberts, 1996).

2.12.1 Classification of tetracyclines

Tetracyclines are classified into two different types' i.e. typical tetracyclines and atypical tetracyclines (Michalova *et al.*, 2004).

2.12.1.1 Typical tetracyclines

A number of semisynthetic tetracyclines belong to the first class of tetracyclines referred to as "typical tetracyclines". This class exibit bacteriostatic activity by means of interacting with bacterial ribosomes and blocking of the protein synthesis (Sum *et al.*, 1998). This class include the following tetracyclines; methacycline, doxycycline, minocycline, rolitetracycline, lymecycline and glycylcyclines (Goldstein *et al.*, 1994).

2.12.1.2 Atypical tetracyclines

Atypical tetracyclines belong to the second class of tetracyclines. They include chelocardin, anhydrotetracycline, anhydrochlortetracycline and thiatetracycline. They exhibit bacteriocidal activity by targeting the cytoplasmic membrane (Oliva *et al.*, 1992; Chopra, 1994). However, due to their low-level inhibition of protein synthesis and their toxicity, these compounds are of no therapeutic interest (Michalova *et al.*, 2004).

2.12.2 Tetracyclines and their uses in veterinary medicine

Tetracyclines are widely used in veterinary medicine mainly for the treatment of gastrointestinal, respiratory and skin, bacterial infectious diseases of locomotive organs and of genito-urinary tract as well as systemic infections and sepsis (Prescott *et al.*, 2000). Due to their activity against a broad spectrum of pathogenic microorganisms;

their absorbity, low toxicity as well as their relatively low cost, has endeared their use in the therapy of animal and human infections as well as for the prophylaxis of infections in food animals (Moellering, 1990; Standiford, 1990).

World production of tetracyclines is estimated to be in thousands of tonnes annually (AHI, 2002). Consumption of tetracyclines antibiotics in veterinary practice is relatively high as compared with other classes of antibiotics (Michalova *et al.*, 2004). Subtherapeutic levels of tetracyclines are used in certain countries as feed additives for growth promotion in animal husbandry (IOM, 1998; Schwartz *et al.*, 1998).

Tetracyclines are probably the most widely used therapeutic antibiotic in food animals because of their broad-spectrum activity and cost effectiveness (Okerman *et al.*, 2004). McEroy (2002) states that tetracyclines account for more than 50 % of all in-feed antibiotics sold for use in food animals in the United Kingdom. Similarly, Van den Bogaard *et al.* (1994) argued that the amount of tetracycline used in farm animals in the Netherlands nearly equaled that of all other antibiotics.

2.12.2.1 Tetracycline as a feed additive

Tetracyclines especially chlortetracycline and other drugs used as feed additives are essential for the purposes of growth promotion and control of diseases in dairy animals (Jenkins and Friedlander, 1982). Chlortetracycline has been used as a feed additive at concentrations ranging from 10-500g per ton of feed (Huber, 1971b). The drug is used to control calf-hood diseases in dairy cattle production (Wallace, 1970).

2.12.2.2 Mode of action of tetracyclines

Tetracyclines permeate through the bacterial cell wall by the passive diffusion and through the cytoplasmic membrane by an energy-dependent process (Franklin and Snow, 1971; Yamaguchi *et al.,* 1991; Tsankov *et al.,* 2003). Antibacterial activity of typical tetracyclines is associated with the reversible inhibition of the protein synthesis (Laskin 1976; Kersten and Frey, 1972). In addition, binding of the drug to the ribosome prevents the attachment of the amino acyl-tRNA to the site "A" of the ribosome. Tetracyclines bind directly to the 30S-subunit protein S7 (Goldman *et al.,* 1983), other ribosomal proteins (S3, S14, and S19) are also involved (Franklin, 1966; Buck and Cooperman, 1990). Similarly, some bases in the 16S-rRNA are important for the binding of the tetracyclines to the ribosomes (Michalova *et al.*, 2004).

2.13 Tetracycline Resistance

The prevalence of bacteria resistance to the tetracycline class of antibiotics has increased following the widespread usage of the compound within clinics, veterinary and agricultural practices (Stead *et al.,* 2007). Shortly after the discovery of tetracyclines, resistance to them was reported (Michalova *et al.,* 2004). *Shigella dysenteriae* was the first tetracycline resistant bacterium discovered and was isolated in 1953 (Watanabe, 1963; Falklow, 1975). Since then, a wide range of tetracycline resistant bacteria strains has been identified. Tetracycline resistance determinants can be found in the genomes of the physiological flora from animals, humans as well as from environmental sources and food. These bacteria can act as a reservoir of resistance genes; transfer these genes to the pathogenic genera which may lead to increasing problems of the treatment of infectious diseases (Chung *et al.,* 1999a; 1999b).

The spread of bacterial resistance according to WHO (2006) has major implications. Under European Union legislation, members of the tetracycline class of antimicrobial compounds are permitted for use in the treatment of bacterial diseases in food-producing animal species. It is therefore important that effective screening and confirmatory procedures are available for detecting tetracyclines in foods of animal origin in order to provide legislators and consumers with confidence that the food products entering the food chain are compliant with the current legislation regarding permissible MRLs.

2.13.1 Acquired tetracycline resistance

Tetracycline resistance in most bacteria is due to the acquisition of new genes, often associated with mobile elements (Roberts, 2005). The genes are usually associated with plasmids and/or transposons and are often conjugative. Currently, there exists 38 different tetracycline resistant (*tet*) and oxytetracycline resistance (*otr*) genes described. These include 23 genes which code for energy- dependant efflux proteins, eleven (11) genes code for ribosomal protection proteins, and 3 genes which code for an inactivating enzyme and one gene with an unknown mechanism of resistance (Roberts, 2005). However, of these 38 *tet* genes, 8 new *tet* genes have been identified and the mechanism of their resistance determined (Billington *et al*., 2002; Anderson *et al.*, 2004).

2.13.2 Tetracycline resistance determinants

Tetracycline resistance determinants are widespread among several bacterial species. They have been identified in 32 Gram-negative and 22 Gram-positive organisms and often found in multidrug resistant bacteria (Roberts, 1996; Levy *et al.*, 1999). According

to Roberts (1996) resistance to any drug is often due to the acquisition of new genes associated with either conjugative plasmids or transposons. Tetracycline resistance occurs by three mechanisms: the use of an energy-dependent efflux of tetracycline, altering the ribosomes to prevent effective binding of tetracycline, and producing tetracycline activating enzymes (Ng *et al.,* 2001).

2.13.3 Types of tetracycline resistant genes

The resistant genes associated with an efflux mechanism are *tet* (A), (B), (C), (D), (G), (I), (M) and (K). The tetracycline resistance genes associated with a ribosomal protection mechanism and/or efflux mechanism are *tet* (K), (L), (M), (O), (S), (P), (Q), (B), (D), (H) and (C). While *tet* (X) is the only example of tetracycline resistance gene causing the enzymatic alteration of tetracycline (Ng *et al.,* 2001). Thirty classes of tetracycline resistance have been identified based on DNA-DNA hybridization with regions from structural genes and DNA sequencing (Tenover *et al.,* 1987; Scott and Rood, 1989; Zhao and Aoki, 1992; Leng *et al.*, 1997).

2.13.4 Identification of new *tet* genes

A number of new genera have been identified carrying previously described *tet* (A), *tet* (B), *tet* (C), *tet* (D), *tet* (G), *tet* (H), *tet* (K), or *tet* (L) efflux genes and/or *tet* (M), *tet* (O), *tet* (S), *tet* (Q), or *tet* (W) ribosomal protection genes (Chopra and Roberts, 2001; Chung *et al.*, 2002). The current information reflects the examination of tetracycline resistance (Tc^r) bacteria from a variety of ecosystems, new species and genera, as well as the continued spread of *tet* over time (Kim *et al.,* 2004). Furthermore, new conjugative transposons, carrying different ribosomal protection *tet* genes, have been identified, and many are related to the Tn *916*-Tn *1545* family of elements (Brenciani *et*

al., 2004; Lancaster *et al.*, 2004;). There are reports on an increase in the percentage of Gram-negative isolates which carry multiple *tet* genes (Wolkerson *et al.*, 2004).

2.13.5 Mechanism of tetracycline resistance

Three different mechanisms of tetracycline resistance have been described (Franklin and Snow, 1971; Burdett, 1986; Speer *et al.*, 1991). All the mechanisms are based on the acquisition of one or several tetracycline resistance determinants, which are widely distributed among bacterial genera (Schnappinger and Hillen, 1996). Additionally, mutations in the rRNA, multidrug transporter systems or permeability barriers may be involved in the resistance to several antibiotics including tetracyclines (Michalova *et al.,* 2004). Furthermore, thirty three different tetracycline resistance (*tet*) genes and three oxytetracycline resistance (*otr*) genes have been described (Roberts, 2003). Moreover, there is no essential difference between the *tet* and *otr* genes, but oxytetracycline resistance genes were first described in oxytetracycline producing animals (Ohnuki *et al.*, 1985; Doyle *et al.*, 1991; Levy *et al.*, 1999).

2.13.5.1 Active efflux proteins

Twenty four (60 %) of all *tet* genes code for energy-dependent membrane associated proteins which exports tetracycline out of the cell (Roberts, 2005). This action reduces the intercellular concentration of tetracycline and protects the bacterial ribosomes *in vivo*. The efflux proteins exchange a proton for a tetracycline-cation complex against a concentration gradient. These genes are the most commonly found *tet* genes in Gram-negative bacteria (Chopra and Roberts, 2001). Efflux of tetracycline is mediated by energy-dependent efflux pumps. Efflux proteins, located in the cytoplasmic membrane exchange a proton for a monocationic magnesium-tetracycline complex. They work as

antiporters and thus reduce the amount of the antibiotic in the cytoplasm (Sum *et al.,* 1998). The regulation of *tet* gene expression differs in Gram-positive and Gram-negative bacteria. In Gram-negative bacteria, each determinant consists of two genes coding for an efflux protein and a repressor protein, both regulated by tetracycline (Michalova *et al.,* 2004). They originated divergently and share the central regulatory region. In the absence of tetracycline, the repressor protein *TetR* binds to the operator of the structural efflux gene and thus blocks its transcription (Hillen and Berens, 1994).

Induction occurs when the Mg^{2+}-tetracycline complex formed in the cell binds to the repressor, and conformation changes of the repressor lead to its release from the operator allowing the transcription of the structural efflux gene. The repressor binds again to the operator if the intracellular amount of tetracycline decreases (Roberts, 1996; Michalova *et al.,* 2004).

2.13.5.2 Ribosomal protective proteins

Ribosomal protection was first discovered in streptococci, and is the second most important mechanism of tetracycline resistance in bacteria after the active efflux (Burdett, 1986). Ribosomal protective proteins ensure the resistance to tetracycline, doxycycline as well as minocycline (Sanchez-Pescador *et al.,* 1988; Taylor and Chau, 1996). Ribosomal protective proteins might confer resistance by means of the reversible binding to the ribosome (Schnappinger and Hillen, 1996). There are eleven *tet* genes coding for ribosomal protective proteins (Saphn *et al*., 2001; Cornell *et al.,* 2003). Under normal conditions, the ribosomes are in standard configuration and function normally. This balance is changed with the introduction of tetracycline into the system (Cornell *et al.,* 2003). The tetracycline binds to the ribosome's configurational state which disrupts the elongation cycle and protein synthesis stops.

The ribosomal protection proteins are believe to interact with the base of h34 protein, within the ribosome, causing an allosteric disruption of the primary tetracycline binding site(s) and the tetracycline molecules are released from the ribosomes (Roberts, 2005). The ribosome returns to its standard conformational state and protein synthesis proceeds. Whether the ribosomal proteins actively prevent tetracycline from binding to the ribosomes after they have been released is not known, nor is it known if once the tetracycline is released whether it can rebind to the same or a different ribosome and inhibit protein synthesis again (Saphn *et al.*, 2001).

2.13.5.3 Enzymatic inactivation

The gene *tet* (X) is the only example of tetracycline resistance due to the enzymatic modification and inactivation of the antibiotic (Speer *et al.,* 1991). This gene has been discovered on two *Bacteroides* transposons, Tn *4531* and Tn *4400*, and has been found to share considerable amino acid homology with a number of NADPH-requiring oxidoreductases (Michalova *et al.,* 2004). The *tet* (X) gene encodes for an NADPH-requiring oxidoreductase, which inactivates tetracycline in the presence of oxygen and NADPH, but has only been found in a strict anaerobe, where oxygen is excluded (Chopra and Roberts, 2001). The *tet* (X) gene has a percentage G+C content of 37.4 % suggesting that it is of Gram-positive ancestry and is active in aerobic *E. coli* (Diaz-Torres *et al.,* 2003).

2.13.5.4 Multidrug-resistance mechanisms

In addition to the specific mechanisms of tetracycline resistance encoded by tetracycline resistance genes, other mechanisms such as the multidrug-resistance can contribute more or less to the resistance to tetracyclines in certain bacteria genera (Michalova *et*

al., 2004). These common mechanisms include mutations, permeability barriers or multidrug transporter systems.

Mutations

The discovery of a mutation in the 16S-rRNA that conferred the resistance to tetracyclines in Gram-positive was reported by Ross *et al.* (1998). This mutation consists in the change of a single base (G-C) at the position cognate with *E. coli* 16S-rRNA base 1058 (Moine and Dahlberg, 1994). Another mutation in the 16S-rRNA was revealed showing a high-level resistance to tetracycline in *Helicobacter pylori*. Identical triple base-pair substitution located in the primary binding sites of tetracycline was discovered by several studies (Geritts *et al.,* 2002; Trieber and Taylor, 2002). However, these substitutions (single and double) mediated only low-levels of tetracycline resistance (Dailidiene *et al.,* 2002).

Permeability barriers

Outer membrane of Gram-negative barrier represents the first effective barrier to the various compounds and this plays a role in the antimicrobial resistance. Porins, the major outer membrane proteins, form channels in the outer membrane and allow the nonspecific passages of small polar molecules, amino acids or nutrients (Nikaido, 1994). The rapid passage of tetracyclines into the cell occurs preferentially via the outer membrane protein F (ompF) and in the magnesium-bound form of tetracycline. Whereas, in the porin-defficient cells the influx of the drug is slow, mainly in its unchanged form (Thanassi *et al.,* 1995). Therefore, the decreased level of ompF synthesis leads to the increased level of tetracycline resistance (Cohen *et al.,* 1989). In addition to the decreased number of porin channels in the outer membrane, several

studies have revealed mutations and amino acid changes that influence the structure and the function of porin (De *et al.*, 2001; Olesky *et al.*, 2002).

Multidrug transporters

Multidrug transporters play important role in tetracycline resistance almost in Gram-negative bacteria (Michalova *et al.*, 2004). On the basis of the energetic criteria, they can be divided into two classes separating multidrug transporters utilizing a proton motive force (PMF) for the exudation of drugs from the cell, and ATB binding cassette (ABC) multidrug transporters that gain the energy for the efflux from the ATP hydrolysis (Paulsen *et al.*, 1996a; Putman *et al.*, 2000).

Within the class of PMF transporters, distinct families of proteins have been distinguished: the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, the resistance nodulation-cell division (RND) family and the multidrug and toxic compound extrusion (MATE) family (Marger and Saier, 1993; Saier *et al.*, 1994; Paulsen *et al.*, 1996b; Brown *et al.*, 1999). The EmrE multidrug transporter (also called Mvrc) of *E. coli* was originally identified on the basis of its ability to confer resistance to ethidium bromide and methyl violgen (Purewall, 1991; Morimyo *et al.*, 1992). The EmrE is a member of SMR family which unifies small efflux proteins that function as drug or proton antiporters and export drugs to the periplasmic space (Nikaido, 1998). Over production of EmrE protein results in the low-level resistance to tetracycline and several other antibiotics (Ma *et al.*, 1994).

2.13.6 Linkages of *tet* genes with mobile elements

The *tet* genes are often associated with plasmids, transposons and conjugative transposons which may carry other antibiotic resistance and/or heavy metal resistance genes (Chopra and Roberts, 2001). Many of these elements code for their own transfer, and may greatly influence their ability to spread to new genera (Roberts, 2005). Integrons have been identified in Gram-negative genera (Chopra and Roberts, 2001), but *tet* genes have not yet been found within integrons, which function as a general gene-capture system, and allow multiple antibiotic genes to be linked (Recchia and Hall, 1995).

A new generation of tetracycline, the glycylcyclines (tigecycline) have been developed to overcome bacterial resistance due to *tet* genes coding for efflux proteins or ribosomal protection proteins (Chopra, 2002; Zhanel *et al.*, 2004). No tigecycline resistant bacteria have been identified in nature, however, it is possible that bacteria carrying acquired *tet* genes may have their *tet* genes mutated and become more resistant to this antibiotic. Therefore, it is unclear how this antibiotic will impact bacterial acquisition and spread of acquired *tet* genes.

It is unlikely that overall use of tetracyclines will change in the near future, especially in countries where tetracyclines are used as growth promoters. Thus the trends will most likely show continued increase in the number of tetracycline resistant genera and the percent of bacterial population no longer susceptible to tetracyclines (Roberts, 2005).

2.13.7 Methods of determining tetracycline resistance in microorganisms

Antimicrobial susceptibility testing for tetracycline resistance can be accurately performed by either dilution or disk diffusion methods and by genetic methods (Jorgensen *et al.,* 1999).

2.13.7.1 Dilution method

Dilution tests results in quantitative minimal inhibitory concentration (MIC) value in microorganisms per milliliter. The MIC is the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test (NCCLS, 2002a). This method is based on the inoculation and growth of the microorganism in media containing different concentrations of an antimicrobial agent. This procedure is done by the agar-based or the broth-based method and the concentration range used depends on the antimicrobial drug and on the microorganism tested (Michalova *et al.,* 2004).

The result obtained is reported as quantitative MIC value in µg/ml and/or as the classification of the microorganism into categories: susceptible, intermediate or resistant, based on the interpretive standards (Jorgensen *et al.*, 1999; NCCLS, 2002b). According to the MIC interpretive standards, recommended by NCCLS (2000a; 2000b), microorganisms other than streptococci are considered to be resistant to tetracycline if MIC $\geq$ 16 µg/ml, intermediate if MIC = 8 µg/ml and susceptible at MIC at $\leq$ 4 µg/ml. While streptococci are considered resistant if MIC $\geq$ 8 µg/ml, intermediate at MIC = 4 µg/ml, while strains are considered susceptible at MIC values $\leq$ 2 µg/ml.

2.13.7.2 Disk diffusion method

This test results in qualitative information about the susceptibility of the microorganism (Schlegelova and Rysanek, 1999). Commercially prepared paper disks impregnated with a defined amount of antibacterial agent are used for this test. The amount for each antimicrobial agent in the disk is standardized (NCCLS, 2000b). The method depends on the diffusion of the drug from the disk and the creation of the concentration gradient in the agar medium surrounding the disk. Disks are applied onto the surface of the agar medium inoculated with a microorganism and after the incubation, the diameter of the zone with suppressed growth is measured (Bauer *et al.*, 1966; NCCLS, 2000b). The recommended interpretive standards by NCCLS (2000ab) include: microorganisms other than streptococci are considered resistant to tetracycline if the diameter of the zone of growth of inhibition is ≤ 14 mm, 15 and 18 mm as intermediate and ≥ 19 mm as susceptible when using a disk impregnated with 30 µg of tetracycline. While for streptococci a zone diameters of ≤18, 19 to 22 and ≥ 23 are considered resistant, intermediate and susceptible respectively.

2.13.7.3 Genetic methods for the detection of antimicrobial resistance genes

Genetic methods may confirm the presence of specific genes conferring tetracycline resistance. However, the presence of genes alone does not necessarily mean resistance of the microorganism, as it is possible (although unlikely) that resistance genes may not be expressed. Genetic methods can be fast and it is possible to use them directly on clinical specimens (Tenover and Rasheed, 1999). The most used methods is PCR (polymerase chain reaction) with specific primers for specific resistance genes; although DNA hybridization, using specific labeled molecular probes is another method for detecting resistance genes. Multiplex PCR using several pairs of primers for several

different resistance genes in a single reaction may allow the detection of more than one resistance genes at a time (Warsa *et al.*, 1996; Ng *et al.*, 2001).

CHAPTER THREE

MATERIALS AND METHOD

3.1 Materials

Sampling bottles, latex hand gloves, Mueller Hinton Agar, Nutrient Agar, Lauryl Sulfate Tryptose Broth(LST), Calcium Containing Buffer (TCN), Biochemical media(Simmon citrate, Urea, Tripple Iron Sugar (TSI), sulfate, Indole, Motility (SIM), Methyl Red (MR), Vogesproskeur (VP), Masking tape, Eosin Methylene Blue (EMB), test tubes, Sterile inoculating loop, Coleman flask, Bijou bottles, Cotton Wool, Petri dishes, Test tube rack, Slanting Rack, Agarose, Eppendorf tubes, Spin columns, Ethidium bromide, DNA Ladder (100bp), Lysis Buffer, Wash Buffer 1and 2, Elution Buffer.

3.2 Equipment:

Detection kit; Microbact 12E kit, Antibiotic discs and Premitest kit, Incubator, Autoclave, Hot plate and Magnetic stirrer, Hot Air Oven, Hood, Fridge, Freezer, Centrifuge, Disc Dispenser, Transilluminator, Vortex machine, GPS Reader, Camera.

3.3 Description of Study Area

Kano State is located in North-Western Nigeria. Created on May 27, 1967, from part of the Northern Region, Kano State borders Katsina State to the north-west, Jigawa State to the north-east, and Bauchi and Kaduna States to the South. The capital of Kano State is Kano, the State originally included Jigawa State which was made a separate state in 1991. It is located between longitude 11°30¹N and latitude 8°30¹E. It has a total area of 20,131Km² and density of 470/km²(1,200/sq ml). It has 44 Local Government Areas and

a population size of 9,383,682 people (National population census, 2007) and cattle population of 580,390 (FDL, 2010).

Historically, Kano State has been a commercial and agricultural State, which is known for the production of groundnuts as well as for its solid mineral deposits. The State has more than 18,684 square kilometres of cultivable land and is the most extensively irrigated State in the country.

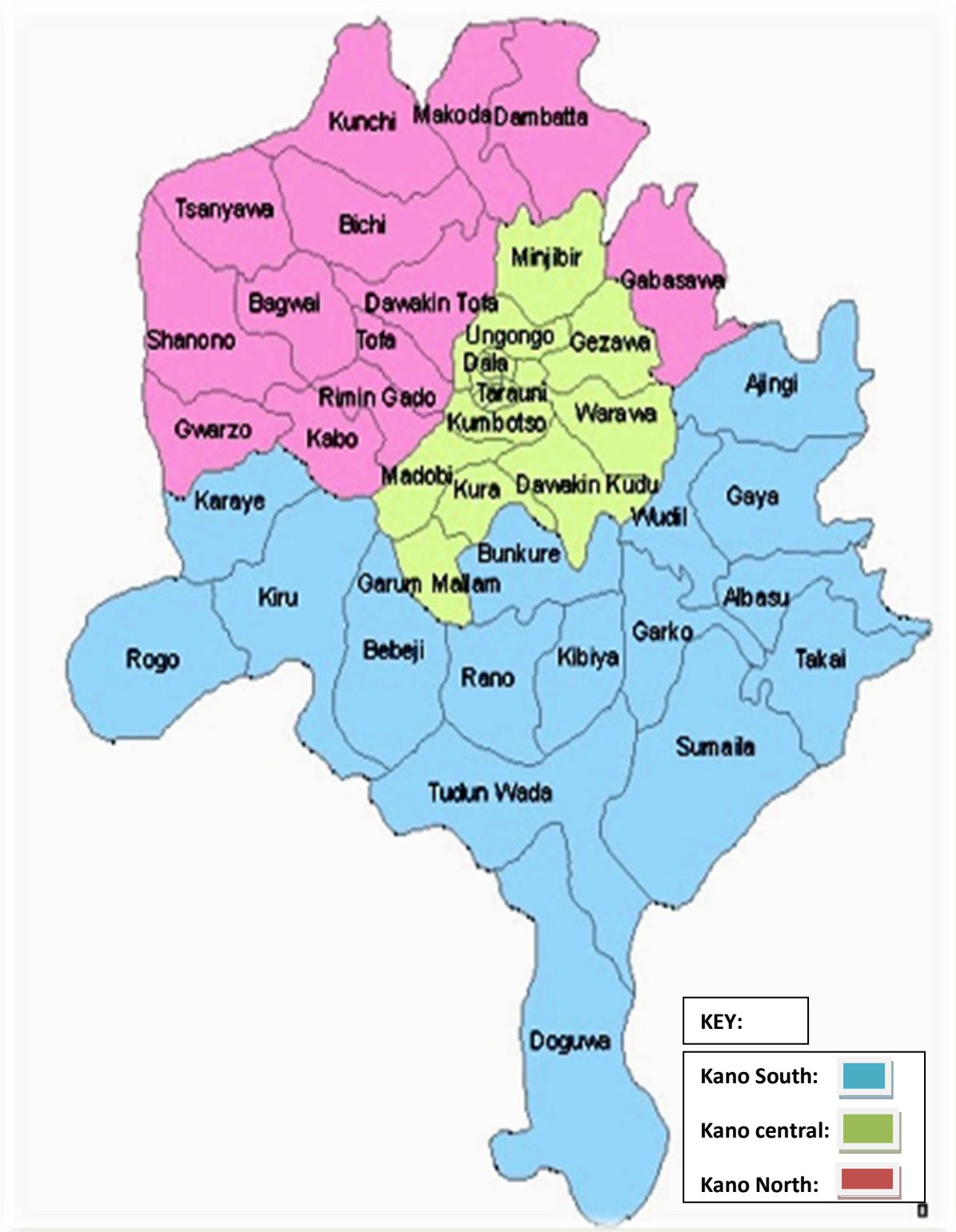


Figure 3.1; Map of Kano State Showing the Senatorial Zones.

3.4 Study Design

The study was a cross sectional study, purposive sampling was carried out between March 2013 to November 2013. Three hundred commercial dairy farms were registered under the commercial dairy project by Kano State agricultural and rural development authority (KNARDA). Eighteen (18%) percent of the total dairy registered farms were sampled giving a total of fifty four (54) farms. This was based on a pilot study that was earlier conducted.

3.4.1 Inclusion criteria

1- Registered farms participating in the commercial dairy project.

2- Farms with a minimum of four milking cows.

3.4.2 Informed Consent

Samples were collected only with the farmer's consent and willingness to participate in the research.

3.5 Sampling Procedure/ Sample Collection

Systematic random sample was employed whereby every 3rd cow in the milking pallor or milking arena was sampled. The animals were sampled according to how they were arranged in the milking pallor sometimes the milking sequence of the farmers was followed. Hand milking procedure was used to extract milk from all the four quarters of the udder with exception of two farms that employed the machine milking process. The udder was thoroughly cleaned and decontaminated with clean water and soap. Also the hands of the milker were washed with chlorhexidine soap. The udder and milker's hands were then dried properly with sanitary towel and each cow was restrained

properly in order to collect the milk sample. The milk was collected from all the four quarters of the mammary glands. Approximately 25 ml of raw milk was collected from each quarter in a sterile sample bottle giving a total of 100 ml per cow. Twenty five percent of the milking cows were sampled from each farm. The milk samples collected were stored on ice packs and transported to the Department of Veterinary Public Health and Preventive Medicine Laboratory, Ahmadu Bello University Zaria, where they were analyzed 4-6 hours after collection.

3.6 Laboratory Analyses

3.6.1 Determination of antimicrobial residues in milk samples.

The milk samples were evaluated for oxytetracycline residues using Premitest kit (R-Biopharm® AG 2013) according to manufacturer's instructions.

Procedure

The required number of ampoules to be used for a particular set of samples were cut open using a pair of scissors, carefully, not to damage the remaining part of the ampoules and about 100µl of the milk sample was pipetted onto the agar in the ampoule carefully not to distort the agar and allowed to stand at room temperature for 20 minutes for pre-incubation.

The test ampoule was washed twice with demineralized water and the last water was removed carefully from the test ampoule. The ampoule was closed with the foil supplied to avoid evaporation and the ampoule was incubated in a Premi test incubator or water bath (64°C± 0.5°C) for 3-4 hours. A negative control (A pre-screened commercially prepared milk) was always set alongside with the tested samples. A positive control

(commercial milk + oxytetracycline which was a HPLC grade Sigma product) was always set for every batch (25 set) of ampoules and the results were read three hours after incubation when the negative control changes color from purple to yellow.

Interpretation of results;

The test results were recorded as either positive or negative. A color change from purple to yellow was termed negative indicating absence of antimicrobial agent while no color change (purple) was termed positive suggesting presence of inhibitory substances or antimicrobial agents. The positive samples on Premi Test were further examined for presence of tetracyclines using a modified PremiTest (a metal iron chelation technique).

3.6.2 Modified Premi Test

TCN buffer (calcium containing buffer)

TCN buffer was use for the modified premitest; Tris Hcl (7.88g) 50mM, calcium chloride (2.19g) 10mM, sodium chloride (8.76g) 150mM dissolved in 1 liter of water and adjusted to pH 7.5 with 1M sodium hydroxide, stored in dark between 4-8°C and used within one month.

STEPS;

For every one hundred (100µl) microliter of the sample, 50µl of TCN buffer was added. It was then vortex- mixed for approximately 30 seconds and allowed to stand at room temperature for 10 minutes. The ampoules were then incubated as in PremiTest and results also recorded as positive or negative.

3.7 Isolation and identification of *E. coli* from milk.

3.7.1 Enrichment of sample

Lauryl sulfate tryptose (LST) broth containing MUG (4-methylumbelliferyl-B-D-glucuronide) was used for the enrichment. It was done according to Manafi (2000). One milliliter (1ml) of the milk sample was dispensed into five (5mls) mls of the broth (LST) in test tubes containing Durham's tubes and incubated for 24 hours at 37°C and observed for fermentation/gas formation in the test tubes. This was evident by the gas that was trapped at the top of the Durham's tubes which pushes the media down.

3.7.2 Plating

About 0.1mls of the incubated sample in broth was streaked on Eosin Methylene Blue (EMB) Agar, with a sterile inoculating loop and incubated at 37°C for 24 hours. Greenish metallic sheen growth was suggestive of *E. coli* which was inoculated on nutrient slants for further biochemical tests.

3.7.3 Conventional biochemical test.

Suspected *E.coli* isolates on EMB were further screened by means of five biochemical tests namely; Simmon citrate, Urea, Tripple Iron Sugar (TSI), sulfate, Indole, Motility (SIM), Methyl Red (MR), Vogesproskeur (VP). Various reactions of the tests such as color change, motility and gas formation were used to interpret results as either positive or negative after 24 hour incubation. These tests were carried out as described in the methodology of Khandaghi *et al*., (2010).

3.7.3.1Triple sugar iron agar test (TSI)

In this test, the Triple Sugar Iron Agar was prepared according to the manufacturer's instruction which was inoculated with the isolates both on the butt and the slant by

stabbing and streaking respectively. This was followed by incubation at 37°C for 24-48 hours. It was then observed for hydrogen sulfide production (which is indicated by a black precipitate at the butt of the tube) and carbohydrate fermentation (indicated by gas production and colour change), (Carter, 1986).

3.7.3.2 Sulphur, indole and motility tests (SIM)

The Sulphide, Indole Motility, SIM media was prepared according to the manufacturer's instruction. The pure isolates were inoculated into the medium by stabbing and incubated at 37^{o}C for 18-24hrs. They were then observed for hydrogen sulphide (H_2S) production, (indicated as a black coloration in the tube) and motility (indicated by migratory movement along the line of stabbing). Three drops of Kovac's indole reagent were then added and shaken gently. After one minute, a positive reaction was indicated by the development of a red color in the reagent layer above the medium which is indicative of *Escherichia coli*.

3.7.3.3 Methyl Red-Voges Proskauer test

Pure isolates of the non-sorbitol fermenters were inoculated into 5ml of MR-VP broth and incubated for 48hrs at 35^{0}C. After incubation, about 1ml of the broth was transferred to a small serological tube followed by the addition of 2-3drops of methyl red and the color on the surface of the medium was read immediately. A red coloration on addition of the indicator signified a positive methyl red test. To the rest of the broth in the original tube, 5drops of 40% potassium hydroxide (KOH) were added followed by 5drops of 5% of alcoholic (ethanol) alpha-Naphthol and shaken. The cap of the tube was loosened and placed in a sloping position. The development of a red color starting from the liquid-air interface within 1hour indicates a positive test. *Escherichia coli* are

reported to be Methyl red positive with an orange to red coloration and Voges-Proskauer negative with no coloration (Cheesbrough, 1985).

3.7.3.4 Citrate utilization test

In this test a sterile needle was used to pick a single isolated colony which were lightly streaked on the surface of the Simmon's citrate agar slant (Prepared according to manufacturer's instruction), which contains a pH indicator (Bromothymol blue) in a test tube (whose screw cap was placed loosely) and incubated at 35^0C for 18-24hours. At neutral pH as a result of organism present not utilizing citrate, a green coloration of the indicator was observed, thus indicating a negative test. *Escherichia coli* are reported to be distinctively citrate negative (MacFaddin, 2000; Reddy, 2007).

3.7.3.5 Urease test

In this test, pure culture was used to streak the entire surface of the urea agar slant prepared in a test tube under sterile conditions. The inoculated test tubes were then incubated for 18-24 hours at 37^oC. Urease production is indicated by a bright pink (fuchsia) color on the slant which identifies those organisms that are capable of hydrolyzing urea to produce ammonia and carbon dioxide, of which *E. coli* is negative for, indicated by the culture medium remaining yellowish in color. (MacFaddin, 2000).

3.8 Microbact 12E Biochemical Test.

Commercially available biochemical test strip Microbact GNB 12E (Oxoid, Uk) was used according to the manufacturer's instructions to confirm isolates as *E.coli* based on the result of the biochemical tests.

Procedure

The isolate to be inoculated was grown on a selective media (EMB). Using a sterile straight wire, 2 to 3 colonies were picked and emulsified in 5.0ml of peptone water and incubated at 37^0 C for 4hours. A sterile pipette was then used to transfer one drop of the peptone water culture into 2.5ml of sterile normal saline solution (0.85%).

The wells of the individual substrate sets were exposed by cutting the end tag of the sealing strip and slowly peeling it back. Using a sterile micropipette, 100µl of the bacterial suspension was added to each well in the set. Mineral oil was then used to overlay the substrates in wells 1, 2 and 3, using a micropipette. The inoculated rows were then resealed and labeled at the end of the tag with the specimen identification number followed by incubation at 37^0 C for 24hours.

On 24hours incubation period, the sealing tape on the test strips were peeled back and evaluated. Results were recorded on a report form as positive or negative by comparing them with a color chart and making reference to the table of reactions provided. However, to well 8 (indole production), two (2) drops of indole (kovacs) reagent was added and the result evaluated within 2 minutes of addition of the reagent. To well 10 (Voges-Proskauer reaction), 1 drop each of VP1 and VP2 reagent was added and the result evaluated within 15 to 30 minutes after the addition. To well 12 (Tryptophan Deaminase), 1 drop of TDA reagent was added and the result evaluated immediately.

Interpretation of result

In Microbact 12E, an octal coding system has been adopted in which each group of three reactions produce a single digit of the code. Using the results recorded on the report forms, the indices of the positive reactions are circled and the sum of these indices in each group of three reactions formed a code of four numbers. The code

obtained was then entered into the computer aided identification package and the resulting identity of the organism and its percentage probability recorded.

3.9 Determination of Antibiotic Susceptibility of *E. coli* Isolates.

Antibiotic susceptibility profile of each isolate was determined using the disc diffusion method according to the CLSI protocol (2006a**)**. Colonies (4-5) of the test isolates from overnight cultures on EMB plates were picked and emulsified in sterile normal saline. The turbidity of the suspension was adjusted to match 0.5 MacFarland's standard. Ten µl of the suspension was then dispensed and spread on Mueller-Hinton agar plates to create a uniform lawn. The pre-inoculated plates were used for the disc diffusion test.

3.9.1 Disc diffusion test.

The isolates were tested with a panel of twelve antibiotic discs (ampicillin 10µg, amoxycillin + clavulanic acid 30µg, chloramphenical 30µg, kanamycin 30µg, gentamicin 10µg, erythromycin 15µg, nitrofurantoin 300µg, tetracycline 30µg, ciprofloxacin 5µg, co-trimoxazole 25µg, cefixime 5µg, nalidixic acid 30µg ,) the antibiotic discs were placed on the surface of each of the pre-inoculated Mueller-Hinton plates using a disc dispenser (Oxoid UK) and the plates incubated aerobically at 37^0 C for 24 hours. After the incubation period the diameters of the antibiotic inhibition zones were measured to the nearest millimeter (mm) using a meter rule and were classified as susceptible (S), intermediate resistant (I) or resistant (R) according to the CLSI (2006b) criteria. Antibiotic discs were obtained from the Oxoid Company (UK).

3.10 Detection of Tetracycline (*tet*) Resistance Genes.

For those *E. coli* isolates found to be resistant to tetracycline, polymerase chain reaction (Multiplex PCR) was used to detect five different types of *tet* genes – *tet* A, *tet* B, *tet* C, *tet* D, and *tet* M. commonly reported in *E. coli* (Chopra and Roberts 2001; Roberts,1996 and Roberts,2005). *E. coli* 16s gene was used as a control.

3.10.1 DNA extraction:

Bacterial DNA was isolated from a 24-hour culture on nutrient slants. The bacterial colonies were suspended in 5ml of LB broth and was taken to DNA Laboratory for extraction.

Steps;

About 1500 µl of the bacterial suspension was centrifuged (Sigma 1-15pk) at 10000rpm for 3 minutes and then the supernatant was discarded and 1000 µl of the sample was added to the Eppendorf tubes and centrifuged (Sigma 1-15pk) again at 10000rpm for 3 minutes and the supernatant was discarded.

Then 300 µl of lysis buffer was added and vortexed to mix properly, 400 µl of lysis buffer was added and set at 80^0 C for 10 minutes in a water bath. The content was transferred into a spin column and centrifuged (Sigma 1-15pk) for 3 minutes at 10000rpm, the content of the collecting column was discarded and 500 µl of wash buffer 1 was added and centrifuged (Sigma 1-15pk) at 10000 rpm for 3 minutes.

The empty column was centrifuged (Sigma 1-15pk) for 3 minutes at 10000 rpm and then transferred to Eppendorf tubes were 60 µl of elution buffer was added into the spin column and incubated for 5 minutes at 80^0 C. It was then centrifuged (Sigma 1-15pk) for 3 minutes at 10000 rpm and 40 µl of the elution buffer was added and recentrifuged

(Sigma 1-15pk). The DNA was then ran on Agarose electrophoresis to characterized the extraction.

The DNA was then used for the detection of the *tet* genes. *E. coli* 16S rRNA gene 832bp (ATCC 25922, accession number x80724, F: TGA CGTTA CCCGCA GAAGAA, R: CTCCAA TCC GGACT ACGACG) and *tet* A gene (300ng/ µl of purified DNA from center for adaptation genetics and drug resistance Boston, USA) were used as positive control and DNASE free water as negative control to validate identity of *E. coli* genes.

3.10.2 Gene amplification conditions.

The PCR assay was performed in 50µl volume containing 0.5µg of extracted DNA as template, 2.5µl PCR buffer mix, 300µM deoxynucleoside triphosphate (dNTPs), primers reported by Ng *et al.* (2001)and $MgCl_2$ was optimized for each multiplexed primer group.The reaction was carried out with an amplification thermal cycler (Applied Biosystem 9700) in repeated cycles of Initial denaturation = 94^0 C for 5minutes followed by 35 cycles of 94^0 C for 1 minute, primer annealing = 55^0 C for 1 minute, Extension = 72^0 C for 1minute 30seconds, and Final extension/elongation = 72^0 C for 10 minutes.

3.10.2.1 Agarose gel electrophoresis:

The PCR products were analyzed by gel electrophoresis using 3g of the agarose (1^{st} base Agarose Biotechnology grade) and diluted with 200ml 1x TAE buffer. The Bands were stained with Ethidium bromide for 5minutes and destained with sterile distilled water for 2-3hours and visualize under translumination. Product size were determine by comparing them with 100bp ladder as above (vivantis #NL1408 LOT 4033-01).

Tetracycline Resistance PCR primers

Plasmid	Tetracycline Resistance genes	PCR Primer sequence 5'-3'	Expected Amplicon size(bp)
pSl18	tet(A)	GCT ACA TCC TGC TTG CCT TC CAT AGA TCG CCG TGA AGA GG	210
pRT11	tet(B)	TTG GTT AGG GGC AAG TTT TG GTA ATG GGC CAA TAA CAC CG	659
pBR322	tet(C)	CTT GAG AGC CTT CAA CCC AG ATG GTC GTC ATC TAC CTG CC	418
pSl106	tet(D)	AAA CCA TTA CGG CAT TCT GC GAC CGG ATA CAC CAT CCA TC	787
pJ13	tet(M)	GTG GAC AAA GGT ACA ACG AG CGG TAA AGT TCG TCA CAC AC	406

3.11 Data Analysis

The data obtained were presented in the form of tables and charts. Chi-square and Fisher's extact test was used to test association between categorical variables where values of $P<0.05$ were considered significant. The analysis was done using IBM Statistical Package for Social Sciences (SPSS version 20).

CHAPTER FOUR

RESULTS

4.1: Occurence Of Antimicrobial Drug Residues From Milk Samples

Out of 313 milk samples tested with Premitest, 79 (25%) were positive for antimicrobial drug residues. Out of 79 milk samples positive for antimicrobial residues, tetracycline residues were confirmed with Modified Premitest in 74 (93.7%) of the milk samples (Figure 4.1).

4.1.1: Association between antimicrobial drug residues by farm type

The antimicrobial drug residues occurred more in the conventional farms (36.51%) than in the cooperatives (17.65%). There was a statistically significant ($P<0.05$) difference in the occurrence of antimicrobial drug residues between the farm types (Table 4.1).

4.1.2: Breed distribution of drug residues by Premitest and Modified Premitest (Oxytetracyclines).

The crosses had the highest percentage of antimicrobial residues 61.11% (22/36) and all of them were tetracycline residues (100%). The Sokoto Gudali had an occurrence of 36.36% (8/22) for antimicrobial residues in which all of them were tetracyclines. Eight (19.51%) out of 41 Friesians that were sampled had antimicrobial residues in their milk in which 3 (37.5%) of them were tetracyclines residues. Two out of the 10 Adamawa Gudali sampled (9.52%) had antimicrobial residues and all of them were tetracycline residues. Antimicrobial residues were detected in 39 out of the 193 Bunaji (20.20%) and all of the residues were tetracyclines. There was a statistically significant association ($p<0.05$) between detection of antimicrobial residue and the breeds of animal sampled. (Table 4.2).

4.1.3: Distribution of residues based on location of farms by Premitest and Modified Premitest.

Occurrence of antimicrobial residues and tetracycline residues were highest in farms further away from the metropolis with occurrence of 30.63% and 100% respectively. Out of 70 samples that were collected from within the metropolis, 19 (27.14%) had antimicrobial residues and 14 (73.68%) were tetracycline residues. Eleven (13.25%) of the 83 samples collected from Local Governments Areas bordering Kano metropolis contained antimicrobial residues and 100% of them are tetracycline residues. There was a statistically significant association ($P< 0.05$) in the distribution of residues between the different locations. (Table 4.3).

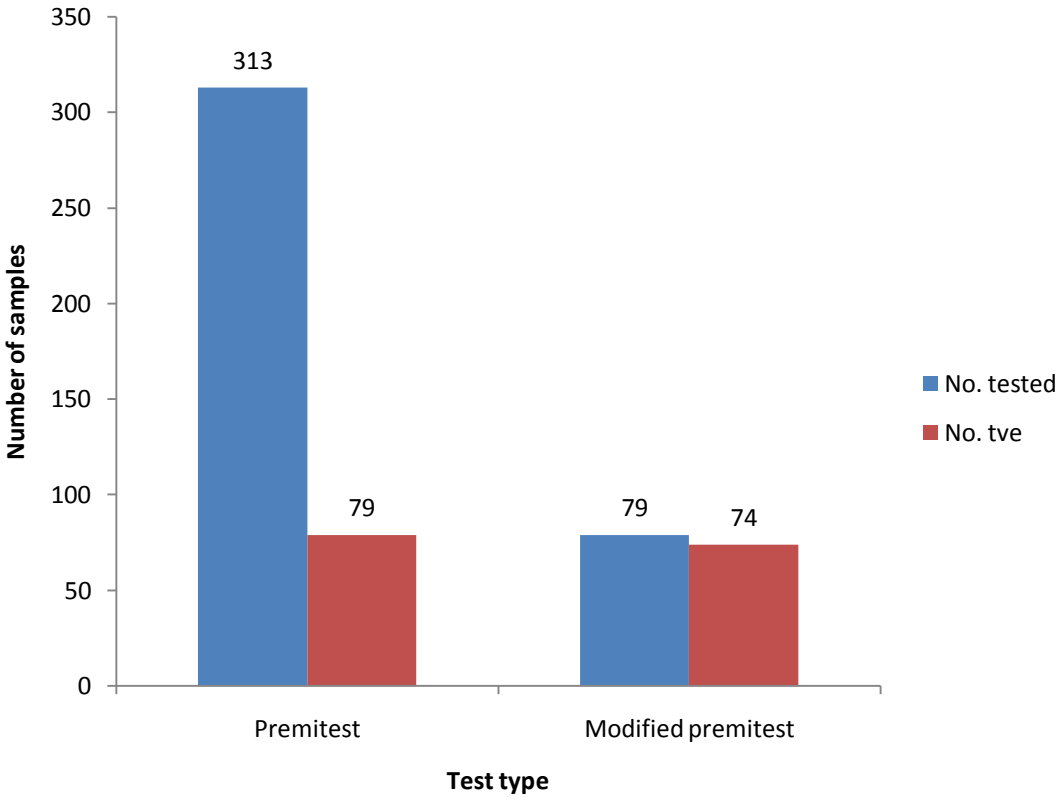


Figure 4.1: Occurrence of Antimicrobial Residues in Milk Samples From Dairy Farms In Kano State.

*Only milk samples positive for Premitest were subjected to Modified Premitest.

Table 4.1: Association Between Drug Residues By Farm Type

Farm type	№ of samples	Positive for Premitest (antimicrobial residues)	Positive for Modified Premitest (tetracycline residues)
Conventional	126	46(36.51%)	41(89.13%)
Cooperative	187	33(17.65%)	33(100%)
Total	313	79(25.24%)	74(93.67%)

Premitest: P= 0.0002, df = 1, χ^2 = 14.192, OR= 2.68, 95% CI= 1.59-4.53

Table 4.2: Breed Distribution of Drug Residues By Premitest (Antimicrobials) And Modified Premitest (Oxytetracyclines).

Breeds	**Total №**	**Premitest (antimicrobial residues)**	**Modified Premitest (tetracycline residues)**
Adamawa Gudali	21	2 (9.52%)	2 (100%)
Bunaji	193	39 (20.20%)	39 (100%)
Friesian	41	8 (19.51%)	3 (37.5%)
Sokoto Gudali	22	8 (36.36%)	8 (100%)
Crosses	36	22 (61.11%)	22 (100%)
Total	313	79(25.24%)	74(93.67%)

Premitest: P = 0.0001, df = 4, χ^2 = 32.04.

Table 4.3: Distribution of Residues Based on Location of Farms by Premitest (Antimicrobial residues) and Modified Premitest (Tetracycline residues).

Location	Total № Tested	Premitest(antimicrobial residues)	Modified Premitest (tetracycline residues)
Category A	83	11 (13.25 %)	11 (100%)
Category B	160	49 (30.63 %)	49 (100%)
Category C	70	19 (27.14 %)	14 (73.68%)
Total	313	87(27.80%)	74(85.06%)

Category A: Bodering larger Kano metropolis, Category B: Further away from Larger Kano metropolis, Category C: Within larger Kano metropolis; Premitest: P = 0.0116, df= 2, χ^2 = 8.914.

4.2: Results of Isolation of *E. coli* From Milk Samples.

Escherichia coli was isolated from 55 (17.57%) out of the 313 milk samples collected and tested. Out of the 55 *E. coli* isolates identified using the conventional biochemical test only, 15 (27.27%) were confirmed to be *E. coli* using the Microbact 12E kit. (Figure 4.2).

4.2.1: Association between *tet* resistance genes and antimicrobial residues.

Out of the 15 *E. coli* confirmed by Microbact, 2 were from tetracycline residue- positive milk and 1 was from tetracycline residue- negative milk. Five of the *E. coli* isolates that were carrying the *tet* genes as confirmed by PCR where from tetracycline residue-negative milk and 7 of the isolates had no *tet* genes and came from tetracycline residue-negative milk. There was no statistically significant association as the p value was greater than 0.05. (Table 4.4).

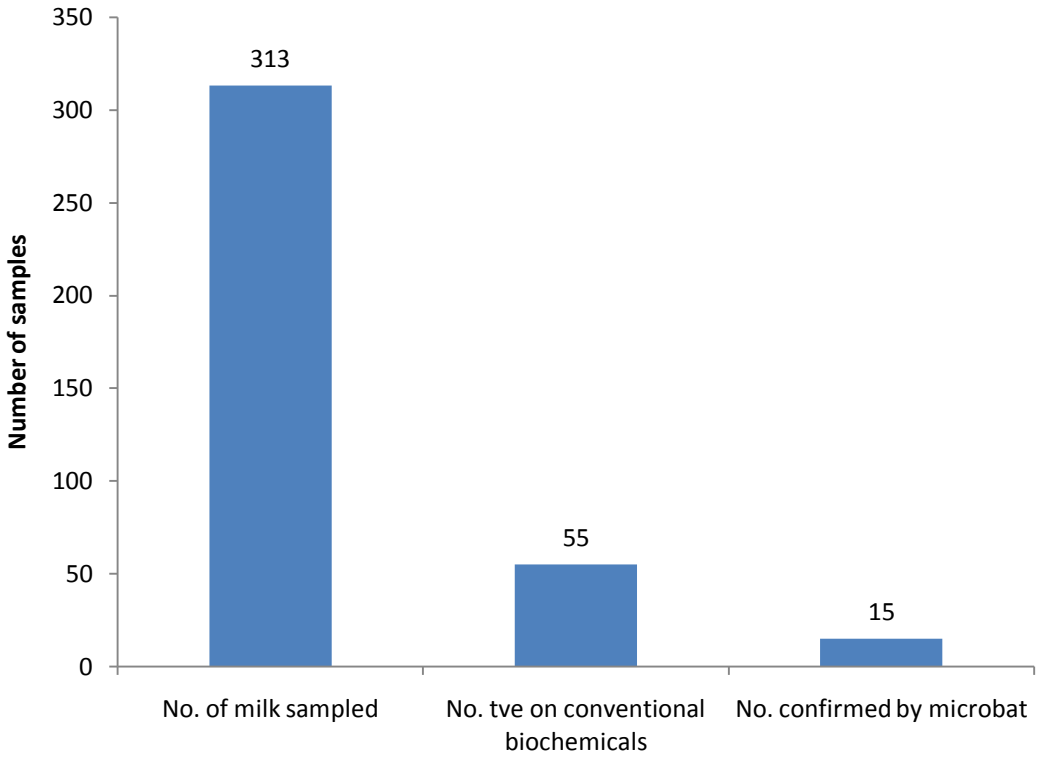


Figure 4.2: Screening of *E. coli* from Milk Samples.

Table 4.4: Association Between *tet* Resistance Genes and Tetracycline Residues.

Tetracycline residues	***tet* genes**	
	№ Positive	№ Negative
Positive	2	1
Negative	5	7
Total	7	8

Fishers's exact test P= 0.569, OR= 2.8, CI= 0.102-188.36

4.3: Antibiotic Resistance Pattern of *Escherichia coli* Isolates.

The relationship between the antibiogram of bacterial isolates with antimicrobial residues was shown in Table 4.5 and Figure 4.3. None of the *E. coli* isolates was resistant to the quinolones (nalidixic acid and ciprofloxacin), All the *E. coli* isolates showed resistance to ampicillin (100%), 73.3% of the *E. coli* isolates were resistant to the macrolide (erythromycin) that was tested and 13.3% of the *E. coli* isolates were resistant to nitrofuran (nitrofurantoin) and cefixime (cephalosporin), 33.3% of the *E. coli* isolates were resistant to amoxycillin+ clavulanic acid (a synthetic penicillin) and co-trimoxazole (sulfa drug). Also 26.7% of *E. coli* isolates were resistant to gentamicin and 40% were resistant to kanamycin (aminoglycosides), 46.7% of the *E. coli* isolates were resistant to tetracycline.

4.3.1: Association of antibiotic profile with antimicrobial residues.

There was a statistically significant difference ($p < 0.05$) between *E. coli* resistance and occurrence of antimicrobial residues. Out of the 4 moderately resistant *E. coli* that were tested 3 (75%) of them were from residue positive milk and 1 (25%) was from residue-negative milk. Ten (90.91%) of the multi-drug resistant *E. coli* were from residue-negative milk. (Table 4.6).

4.3.2*: Esherichia coli* isolates and multiple antibiotic resistance indices.

The antibiograms were used to calculate the multiple antibiotic resistance indices, 73.3% of the *E. coli* isolates had MAR value greater than 0.2. The multiple antibiotic resistance indices of the bacterial isolates was displayed in Table 4.7 and Figure 4.4, It was calculated by dividing the total number of antibiotics to which an isolate was resistant by total number of antibiotics tested (12).

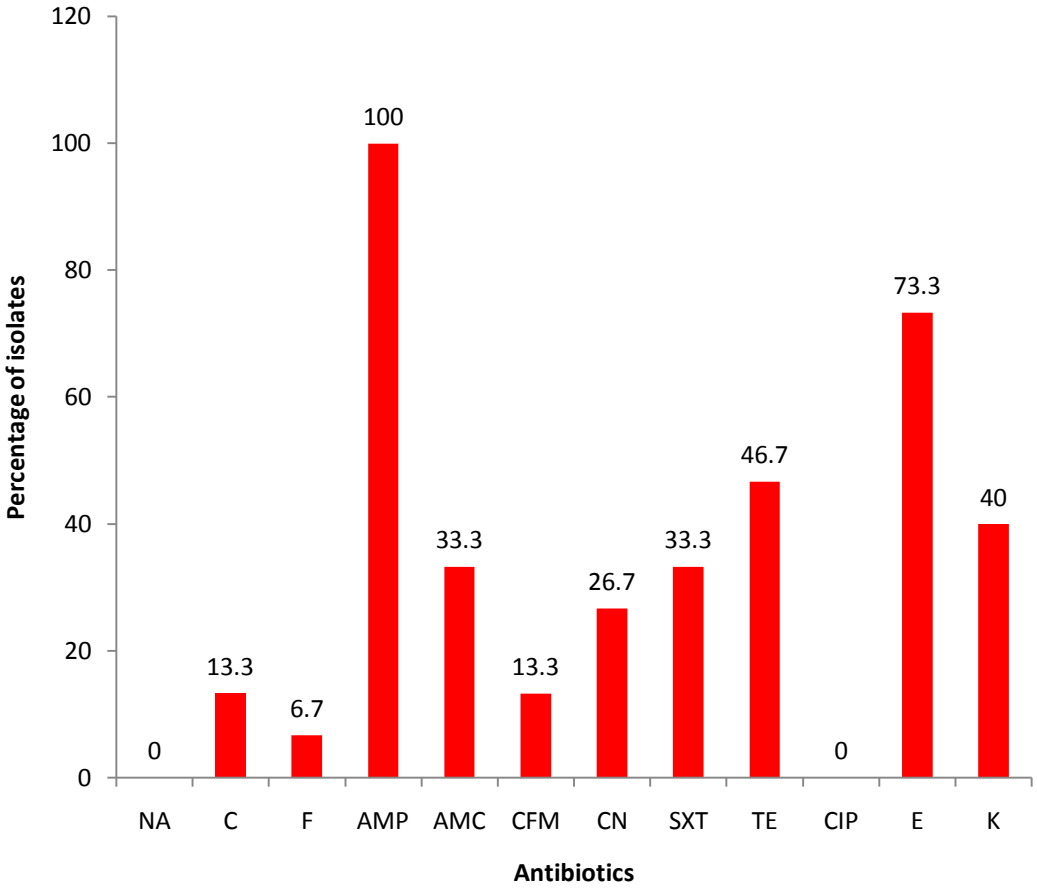
4.3.3: Multiplex PCR for determination of tetracycline resistance genes.

Plate I shows the agarose gel electrophoresis of PCR products of the *E.coli* resistant isolates. The PCR result showed that 6 (40%) of the isolates were carrying *tet* A gene and 1 isolate carried both *tet* M and *tet* A genes. This isolate (lane 10) displayed multiple resistance on disc diffusion test and was isolated from residue positive milk.

Table 4.5: Antibiotic Resistance Patterns of *Escherichia coli* Isolates From Milk of Dairy Cows In Kano State.

S/N	Resistance Profile
1	AMP,TE,K,E,
2	AMP,AMC
3	AMP,TE,E
4	AMP,AMC,SXT,K,E,C
5	AMP,AMC,TE,K,CN,E
6	AMP,AMC,CFM,TE,SXT,K,E,F
7	AMP,K,CN,E
8	AMP,CN,E
9	AMP
10	AMP,AMC,SXT,E
11	AMP,SXT,E
12	AMP,AMC,TE,E
13	AMP,CN
14	AMP,TE
15	AMP,CFM,TE,SXT,K,E,C

AMP: Ampicilin, TE: Tetracycline, K: Kanamycin, E: Erythromycin, AMC: Amoxycillin + Clavulanic acid, SXT: Co-trimoxazole, C: Chloramphenical, CN: Gentamicin, CFM: Cefixime, F: Nitrofurantoin.



AMP: Ampicilin, TE: Tetracycline, K: Kanamycin, E: Erythromycin, AMC: Amoxycillin + Clavulanic acid, SXT: Co-trimoxazole, C: Chloramphenical, CN: Gentamicin, CFM: Cefixime, F: Nitrofurantoin.

Figure 4.3: Percentage Resistance of *E. coli* isolates to various Antibiotics.

Table 4.6: Association of Antibiotic Profile Pattern with Antimicrobial Residues.

Antibiotic Profile Pattern	**№ Tested**	**Negative (%)**	**Positive (%)**
Moderate Resistance[a]	4 (26.67%)	1 (25 %)	3 (75 %)
Multiple Resistance[b]	11 (73.33 %)	10 (90.91 %)	1 (9.09 %)
Total	15 (100%)	11 (73.33 %)	4 (26.67%)

Fisher's exact test*P = 0.033, df = 1; a: resistance to 1 or 2 antibiotics, b: resistance to more than 2 antibiotics. CI = 0.0016-0.710.

Table 4.7*: Esherichia coli* Isolates and Multiple Antibiotic Resistance Indices (MAR).

S/N	MAR
1	0.33
2	0.17
3	0.25
4	0.5
5	0.5
6	0.67
7	0.33
8	0.25
9	0.08
10	0.33
11	0.25
12	0.33
13	0.17
14	0.17
15	0.58

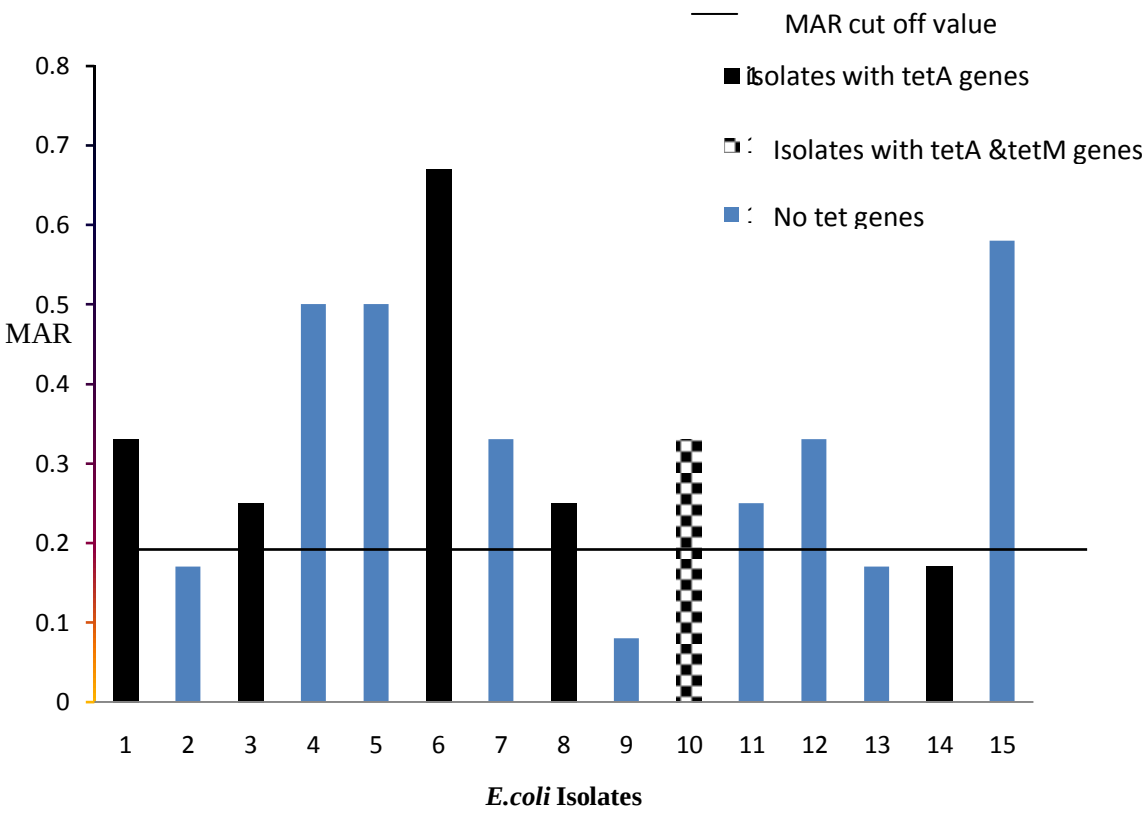


Figure 4.4: Multiple Antibiotic Resistance Index of the *E. coli* Isolates.

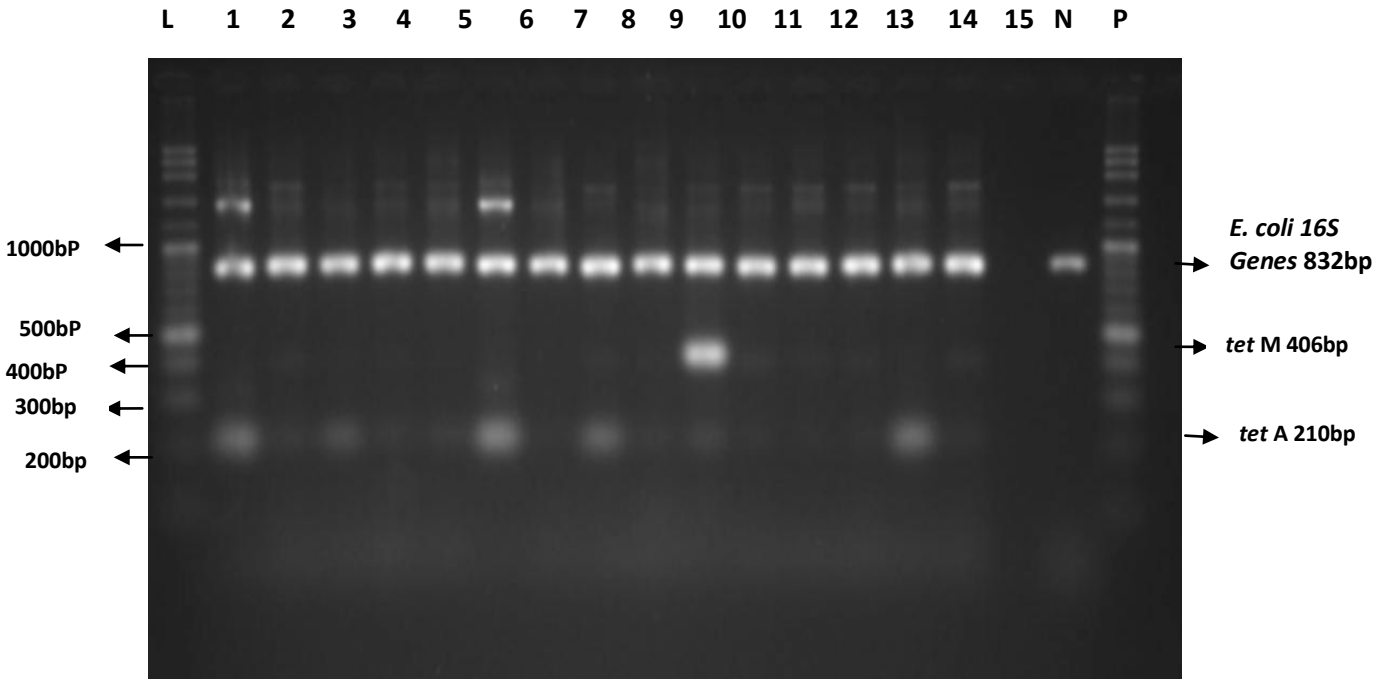


Plate I : Multiplex PCR result displaying *tet* M (406bp) at lane10, *tet* A(210bp) at lanes 1,3,6,8,10 and 14,and *E.coli*16s genes. L represents 100bp laddder, N= Negative control and p= positive control.

CHAPTER FIVE

DISCUSSION

This study found the overall occurrence of antimicrobial residues to be 25% out of which 93.7% were due to tetracyclines. High occurrence of antimicrobials and tetracycline residues in dairy cows, goats and various animal tissues was also reported by Van-Dresser and Wilcke, (1989); Kabir, (1998) Dipeolu and Alonge, (2001); Kabir *et al.*, (2004); Dipeolu and Dada (2005); Olatoye and Ehinmowo, (2009); Movassagh, (2011); Olatoye *et al*., (2011); Darwish *et al*., (2013); Ezenduka *et al*.,(2014). However some other researchers found low levels of tetracycline residues in milk and animal tissues (Navratilova *et al*., 2009; Adetunji and Olaoye, 2012; Tona and Olusola, 2014). Bane *et al*., (1989) reported that the type of on-site screening test used in a farm could determine how frequent residues may be found. Premitest was used in this study to screen for residues and this test detects antibiotics above the MRL or tolerance levels set up by W.H.O of 0.1mg/kg in milk. The result of this study implies that a significant percentage of the milk produced by commercial dairy farmers in Kano State is contaminated which will affect its quality, marketability and make it unsafe for human consumption. The farmers probably do not observe drug withdrawal period before milking and most likely there is profuse use of antimicrobial agents by the farmers particularly, tetracyclines. Van-Dresser and Wilcke (1989), reported that presence of antibiotic residues results from injudicious use of drug preparations and these are a function of sales volume of the drugs.

The occurrence of antimicrobial residues varied between the farm types with a statistically significant difference ($p<0.05$). The conventional farms had the highest occurrence of antimicrobial residues (36.51% of which 89.13% were tetracyclines)

compared to the cooperative farms (17.65% antimicrobial residues and all (100%) were tetracycline). This suggests that the conventional farms probably use more antibiotics than the cooperatives because of the operation type. This group of farmers can afford to buy the antibiotics and operate a more intensive system hence the high usage. Previous researchers Kaneane and Alwynelle, (1987); Mc Ewen *et al.*, (1991); Wilson *et al*, (1991); Van-Dresser and Wilcke (1989) reported that management factors are perhaps the most important factors that influence the occurrence of antimicrobial residues.

Also, occurrence of antimicrobial residues differed between the various breeds that were sampled. There was a statistically significant association ($p<0.05$) between detection of antimicrobial residues and the breeds of animals sampled. The crosses had the highest occurrence of antimicrobial residues (61.11%) of which all were tetracyclines (100%), followed by Sokoto Gudali with 36.36% (100% tetracyclines). These are the two major breeds kept by the conventional farms sampled and this group had the highest occurrence of antimicrobial residues in milk. The Adamawa Gudali and Friesian had a lower occurrence (9.52% and 19.51% respectively) compared to Bunaji (20.20%) despite being kept by the conventional farms because they are higher milk producing animal compared to Bunaji. Siddique *et al.*, (1965) reported that the rate of disappearance of drugs from milk is slower in dairy cows with lower milk production (volume) than high producers. However the role of confounding in this relationship cannot be overruled.

The occurrence of residues was found to differ from one location to another with a statistically significant difference ($p<0.05$). The problem of residues in milk in Kano State is wide spread. Guest and Paige, (1991) and Wilson *et al.*, (1991) reported that geographical distribution i.e variation from region to region and seasonal patterns

(weather and climate) can influence occurrence of antimicrobial residues. Farms located in Local Government Areas further away from Kano metropolis had the highest residue occurrence (30.63%) and all of the residues were tetracyclines (100%). The inaccessibility of these farms to social services, good veterinary care and health programs make them prone to inappropriate drug usage and treatment of their own animals. This is in agreement with the findings of Movassagh (2011) in Iran who also found variation in distribution of drug residues between various places he sampled. Farms located within the larger Kano metropolis had high residue occurrence too, probably because they have easy access to over the counter drugs hence high antibiotic usage by these farms. Farms located at Local Government Areas bordering larger Kano metropolis had relatively low residue levels may be because these farms are the first set of the peri-urban dairy registered farms, they enjoy better monitoring and supervision.

This study found low occurrence of *E. coli* in milk samples and the low occurrence could be associated with drug residues in the milk samples in which significant percentage of them are tetracyclines (75%). All the *E. coli* isolates displayed resistance to one form of antibiotic or another on disc diffusion test. Twenty percent of the *E. coli* that were isolated came from residue- positive milk while 80% were from residue-negative milk. Seventy five percent (75%) of the moderately resistant *E. coli* were from residue positive- milk and 90.91% of the multidrug resistant *E. coli* were from residue-negative milk. This means that occurrence of residues did not influence multi-drug resistance. Also there was no statistically significant association between occurrence of tetracycline residues and the *tet* genes isolated ($p>0.05$). Contrary to this study, previous studies of concurrence of *E.coli* and antimicrobial drug residues in milk indicated high level resistance by *E.coli* isolates as a result of the concurrence (Abramson, 2007 and Sawant *et al*., 2007). The *E. coli* isolates had Multiple Antibiotic Resistance Index

(MAR) greater than 0.2 about 73.3% of the isolates, this suggests that they were isolated from environment where antibiotics are probably abused or frequently used. The resistance pattern varied between various classes and within the different classes of antibiotics. None of the *E. coli* isolates was resistant to any of the quinolones tested (nalidixic acid and ciprofloxacin) Nosofor and Iroegbu, (2012) attributed low level resistance of quinolones to low usage in livestock therapy in Nigeria due to their relatively higher cost and are not readily available compared to other classes of antibiotics, 13.3% of the isolates were resistant to chloramphenicol (a synthetic antibiotic) and cefixime (a cephalosporin), this implies resistance spread to broad spectrum antibiotics and a new generation antibiotic which will make treatment difficult. Also, 33.3% of the isolates were resistant to amoxicillin+clavulanic acid (a synthetic penicillin) and co-trimoxazole (a sulfa drug), this resistance pattern most likely is due to the integron system of multidrug resistance mechanism in most cases of sulfa drug resistance, integron systems are involved (Kadlec *et al*., 2011). Furthermore, 26.7% and 40% of the isolates were resistant to gentamicin and kanamycin (aminoglycosides) respectively. Resistance to tetracycline was 46.7% and all of the isolates were resistant to ampicillin (a synthetic penicillin) and in this case there is the likelihood that efflux pump system of drug resistance is involved as it gives room for resistance to different classes of drugs at the same time. Similar high level resistance to ampicillin by *E.coli* isolated from milk was reported by Abramson, (2007).

The PCR result showed that 6 (40%) of the isolates carried *tet* A gene which codes for efflux pump and 1 isolate carried *tet* M gene which codes for both efflux pump and ribosomal protection resistance mechanisms (see plate i) and this supports the multidrug resistance displayed by most of the *E. coli* isolates as these two resistance mechanisms gives room for the multidrug resistance. This isolate displayed multiple resistance on

disc diffusion test and was isolated from residue positive milk. The *tet* A and *tet* M genes are the most common resistance mechanisms of *E. coli* to tetracyclines as reported by Roberts, (2005) and the findings of this research suggests that *tet* A and *tet* M are the predominant resistance genes of the *E.coli* isolates in the study area.

In the course of this research, 40 other bacterial isolates where identified by the Microbact 12E system. Twenty seven (67.5%) of the isolates displayed multiple drug resistance, 2 (5%) of the isolates were susceptible to all the drugs tested and 11 (27.5%) of the isolates had moderate resistance profile. Also 10 (25%) of the isolates were from tetracycline positive milk. This suggests transmissible resistance and role of mobile genetic elements between members of *Enterobacteriacae*.

CHAPTER SIX
CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

This study determined the occurrence of antimicrobial residues in milk samples from commercial dairy farms in Kano State Nigeria at 25% with high occurrence of tetracyclines (93.7%). The study showed that occurrence of antimicrobial residues could vary by breed, farm type and location of the farm. All the *E.coli* that were isolated and identified were resistant to one or more of the antibiotics tested. It also showed that animals shedding residues in milk are not likely to be shedding multi-drug resistant *E. coli* and 73.3% of the isolates probably came from farms where antibiotics were abused or frequently used. The study further revealed that some of the *E. coli* isolates carried genes that codes for both efflux pumps and ribosomal protection resistance mechanisms.

6.2 Recommendations

1. The farmers should be enlightened on the dangers of antimicrobial residues in milk and risk of possible losses that could arise from limited market prospects.
2. There should be good regulatory policy by government on the use and sales of antimicrobials in animal production in the country.
3. Quality veterinary service provision by government is needed to curb issues of quackery and drug abuse in the industry.
4. Farmers should observe drug withdrawal period before milking their animals and they should adopt the use of on-farm screening methods to screen residue containing milk in their farms.
5. Further studies should be conducted on such farms using more sensitive and specific residue detection methods like HPLC.

6. There is need to further detect other resistant genes apart from *tet* genes as the isolates could be carrying them.

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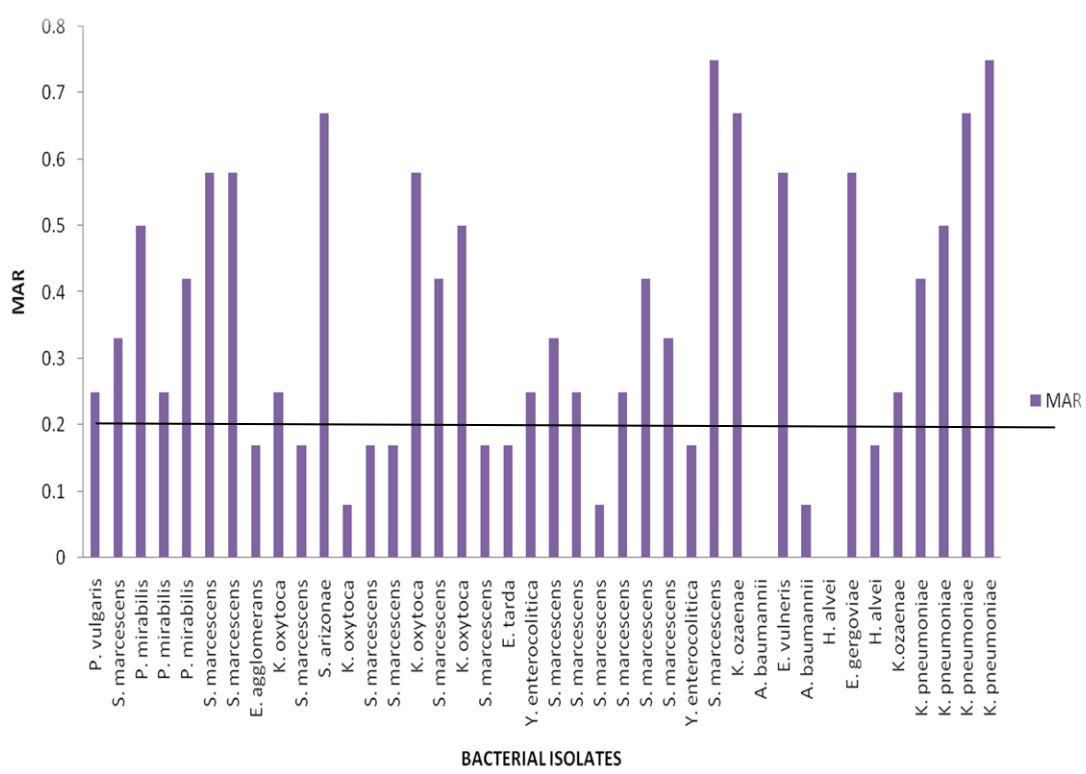
Appendix

Appendix 1: Profiles of Bacterial Isolates and Relationship with Antimicrobial Residues.

Isolates	Antibiotic Profile	Premitest	M.Premitest
P. vulgaris	AMP,AMC,CIP	NEGATIVE	
S. marcescens	AMP,TE,E,C	NEGATIVE	
P. mirabilis	AMP,TE,SXT,K,E,C	POSITIVE	NEGATIVE
P. mirabilis	AMP,TE,E	NEGATIVE	
P. mirabilis	AMP,TE,E,F,C	NEGATIVE	
S. marcescens	AMP,TE,SXT,K,E,F,C	NEGATIVE	
S. marcescens	AMP,TE,K,E,C,NA,CIP	POSITIVE	POSITIVE
E. agglomerans	AMP,AMC	NEGATIVE	
K. oxytoca	AMP,TE,E	POSITIVE	POSITIVE
S. marcescens	AMP,E	NEGATIVE	
S. arizonae	AMP,AMC,TE,SXT,K,CN,NA,CIP	NEGATIVE	
K. oxytoca	TE	NEGATIVE	
S. marcescens	AMP,E	NEGATIVE	
S. marcescens	AMP,AMC	NEGATIVE	
K. oxytoca	AMP,CFM,TE,SXT,K,CN,E	NEGATIVE	
S. marcescens	AMP,CFM,E,F,NA	NEGATIVE	
K. oxytoca	AMP,AMC,TE,K,CN,E	NEGATIVE	
S. marcescens	AMP,E	NEGATIVE	
E. tarda	AMP,E	NEGATIVE	
Y. enterocolitica	AMP,TE,E	NEGATIVE	
S. marcescens	AMP,AMC,TE,E	NEGATIVE	
S. marcescens	AMP,TE,E	NEGATIVE	
S. marcescens	E	NEGATIVE	
S. marcescens	AMP,TE,E	NEGATIVE	
S. marcescens	AMP,TE,SXT,E,C	POSITIVE	POSITIVE
S. marcescens	AMP,TE,SXT,E	POSITIVE	POSITIVE
Y. enterocolitica	AMP,E	NEGATIVE	
S. marcescens	AMP,AMC,TE,K,CN,E,F,C,CIP	POSITIVE	POSITIVE
K. ozaenae	AMP,AMC,CFM,TE,SXT,K,CN,NA	POSITIVE	POSITIVE
A. baumannii		NEGATIVE	
E. vulneris	AMP,AMC,CFM,SXT,E,C,NA	POSITIVE	POSITIVE
A. baumannii	AMP	NEGATIVE	
H. alvei		NEGATIVE	
E. gergoviae	AMP,AMC,CFM,TE,K,E,NA	POSITIVE	POSITIVE
H. alvei	AMP,AMC	NEGATIVE	
K.ozaenae	AMP,SXT,E	POSITIVE	POSITIVE
K. pneumoniae	AMP,AMC,CFM,TE,SXT	NEGATIVE	
K. pneumoniae	AMP,AMC,CFM,TE,SXT,E	NEGATIVE	
K. pneumoniae	AMP,AMC,CFM,TE,SXT,E,F,C	NEGATIVE	
K. pneumoniae	AMP,AMC,CFM,TE,SXT,K,CN,E,CIP	POSITIVE	POSITIVE

AMP: Ampicilin, TE: Tetracycline, K: Kanamycin, E: Erythromycin, AMC: Amoxycillin + Clavulanic acid, SXT: Co-trimoxazole, C: Chloramphenical, CN: Gentamicin, CFM: Cefixime, F: Nitrofurantoin, NA: Nalidixic Acid, CIP: Ciprofloxacin.

Appendix 2: Multiple Antibiotic Resistance Index of the Bacterial Isolates.



Appendix 3: Percentage Resistance of Bacterial Isolates to Various Antibiotics

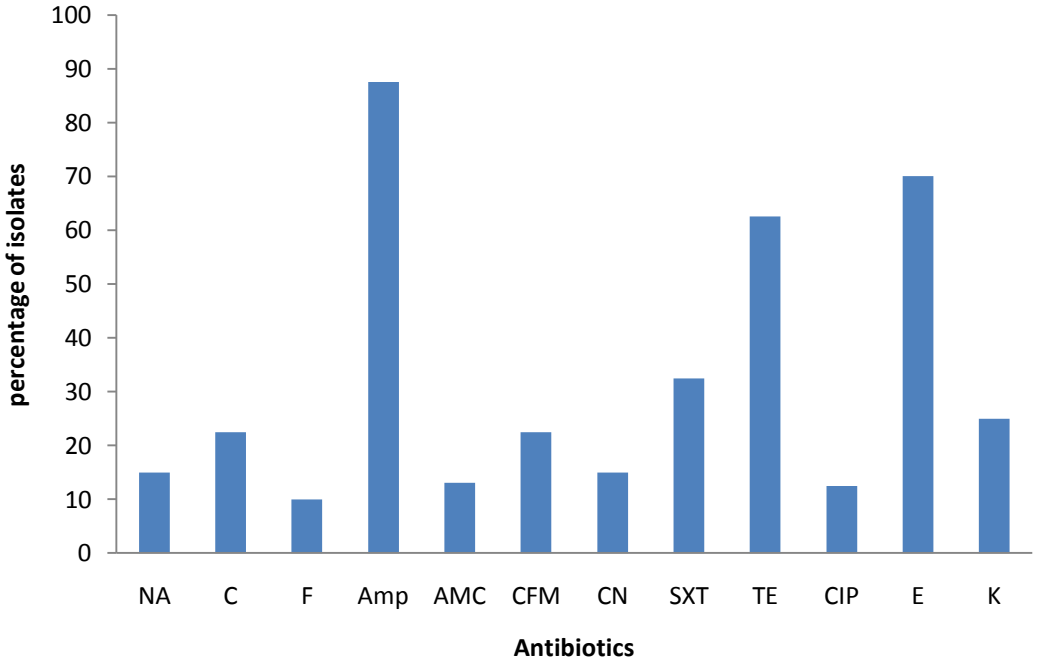


Plate II: Machine Milking.

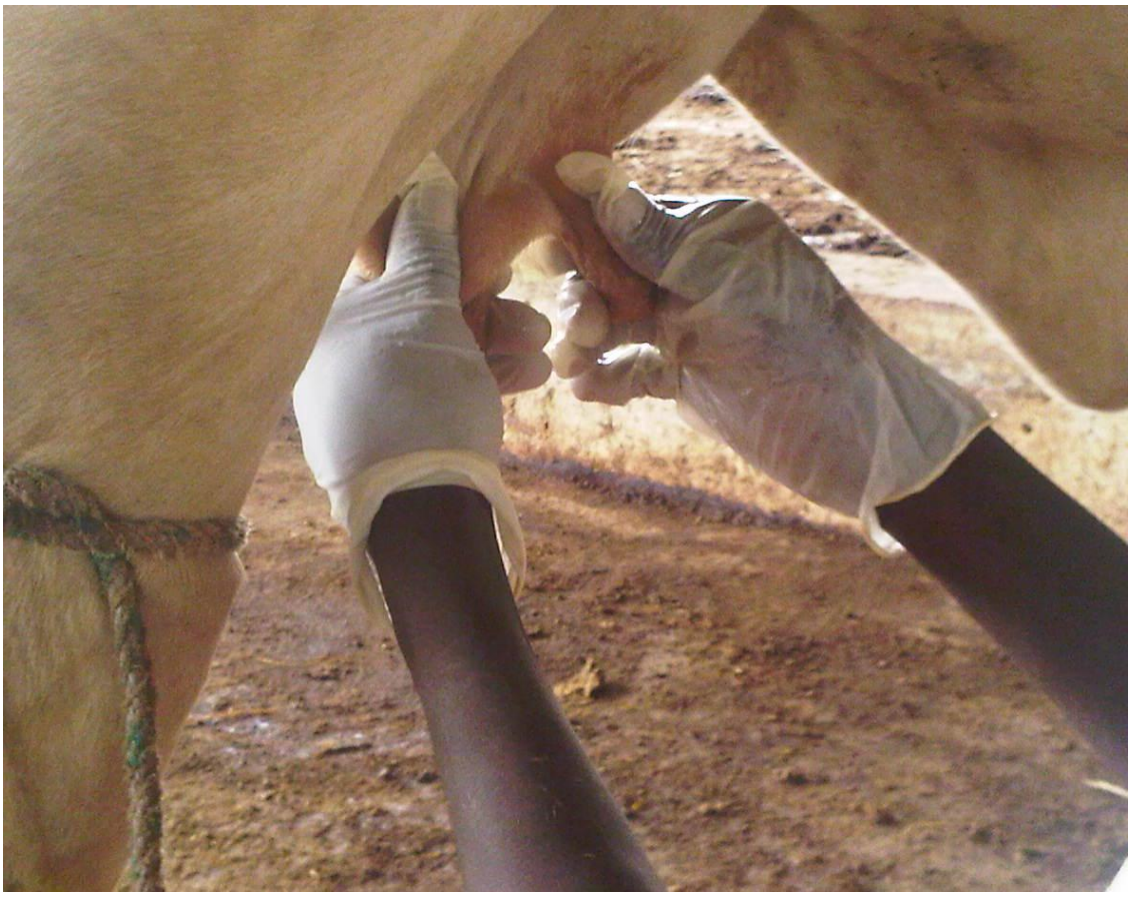
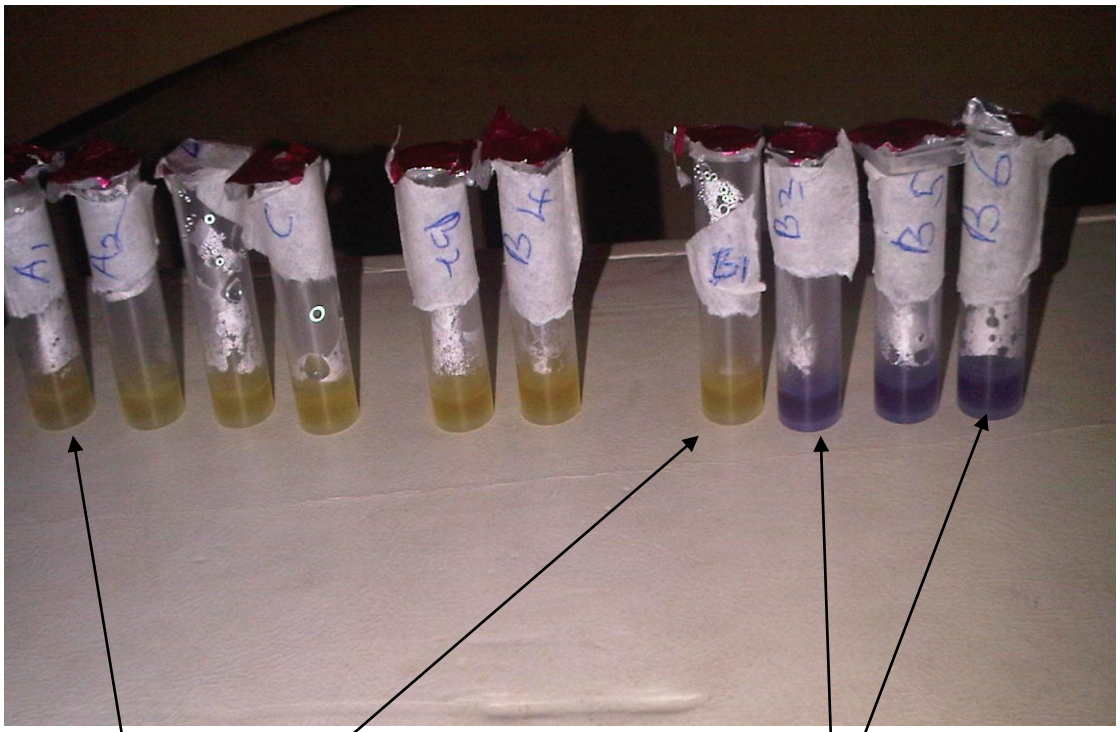


Plate III: Hand Milking.

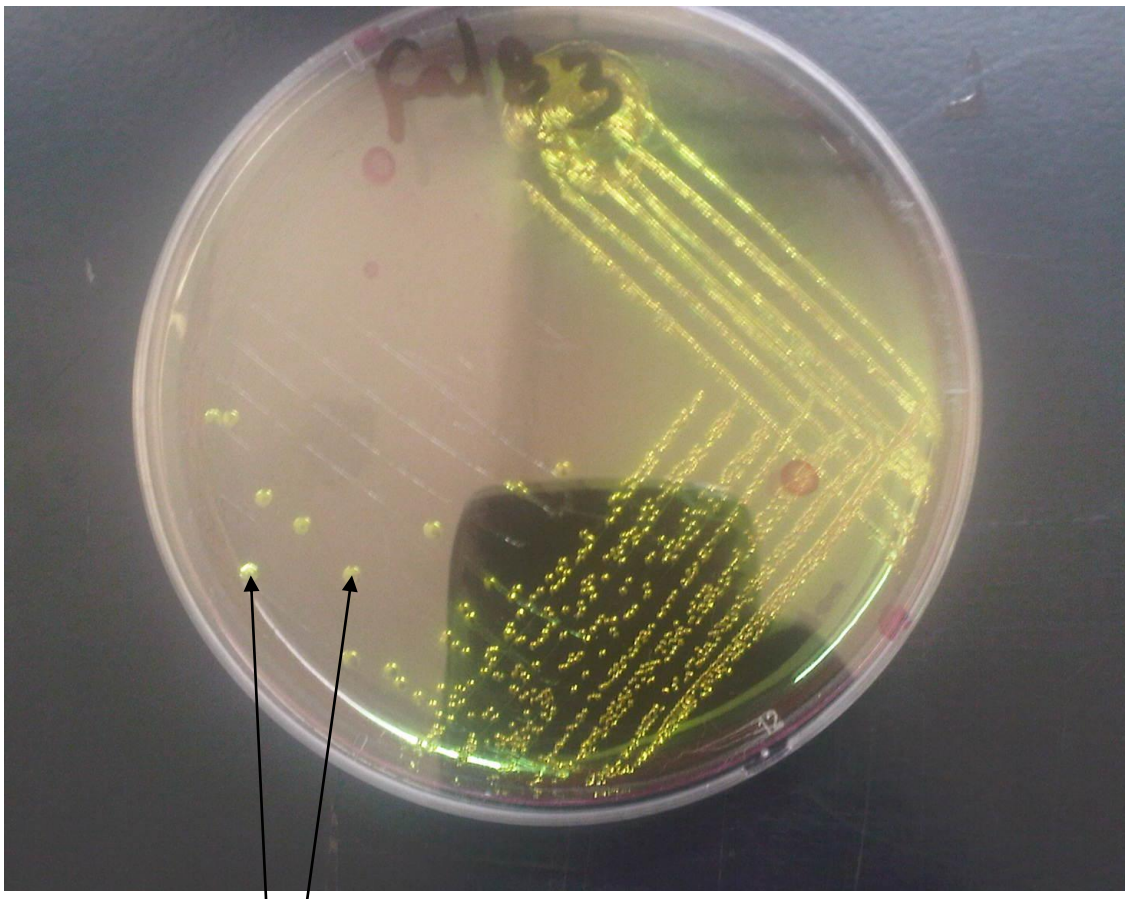
Plate IV: Premitest Incubator with Agar Ampoules Loaded in the Wells.



Positive results.

Negative results

Plate V: Premitest Results for Detection of Antimicrobial Residues.



Discreet *E.coli* colonies

Plate VI: Suspected *E.coli* Isolates on E.M.B Plate.

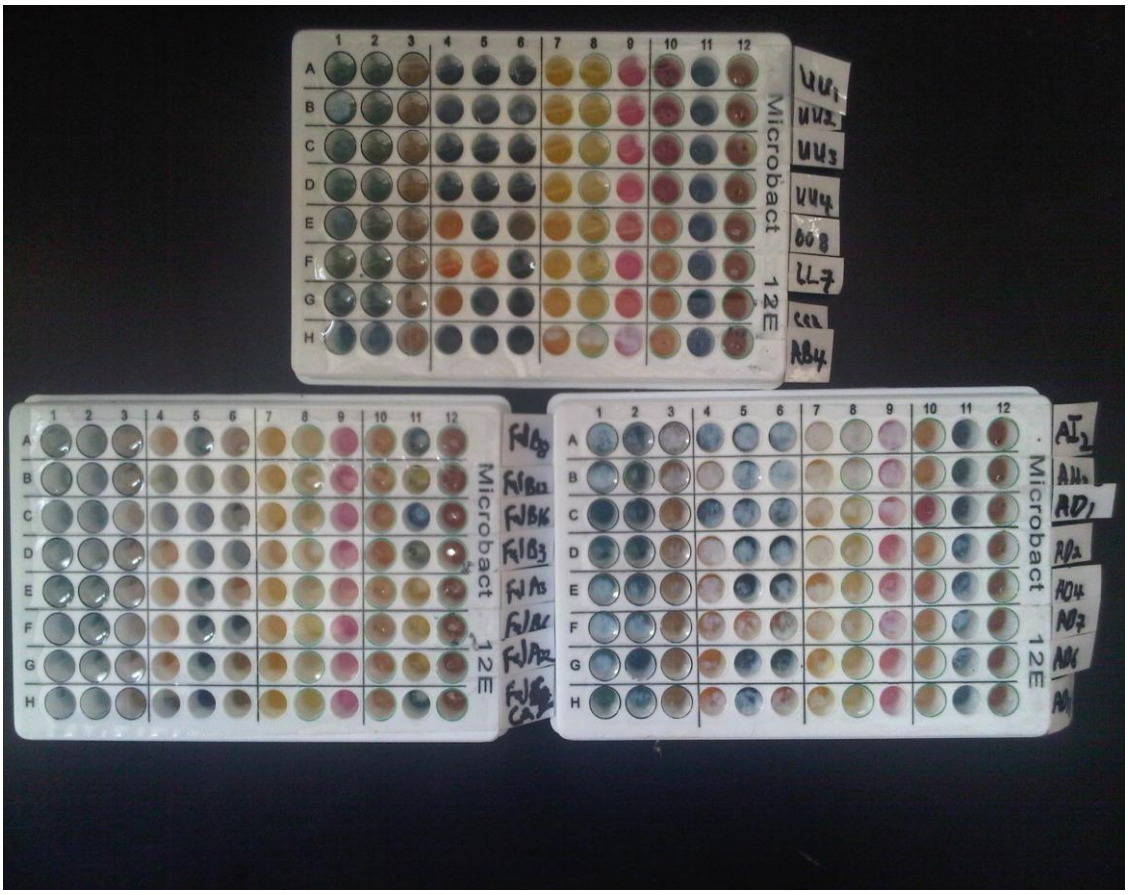


Plate VII: MicroBact 12E Plates After 24 Hour Incubation.

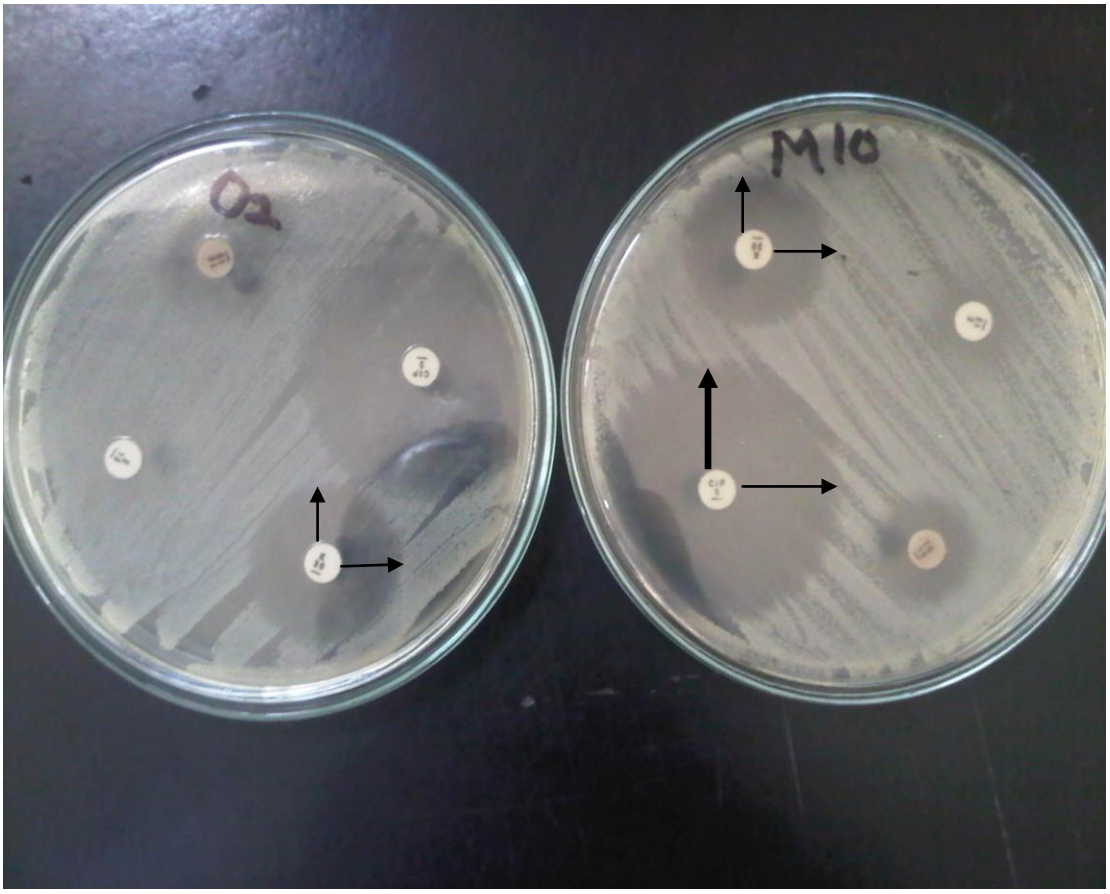


Plate VIII: Disc Diffusion Test with Arrors Showing Zones of Inhibition of Bacterial Growth.

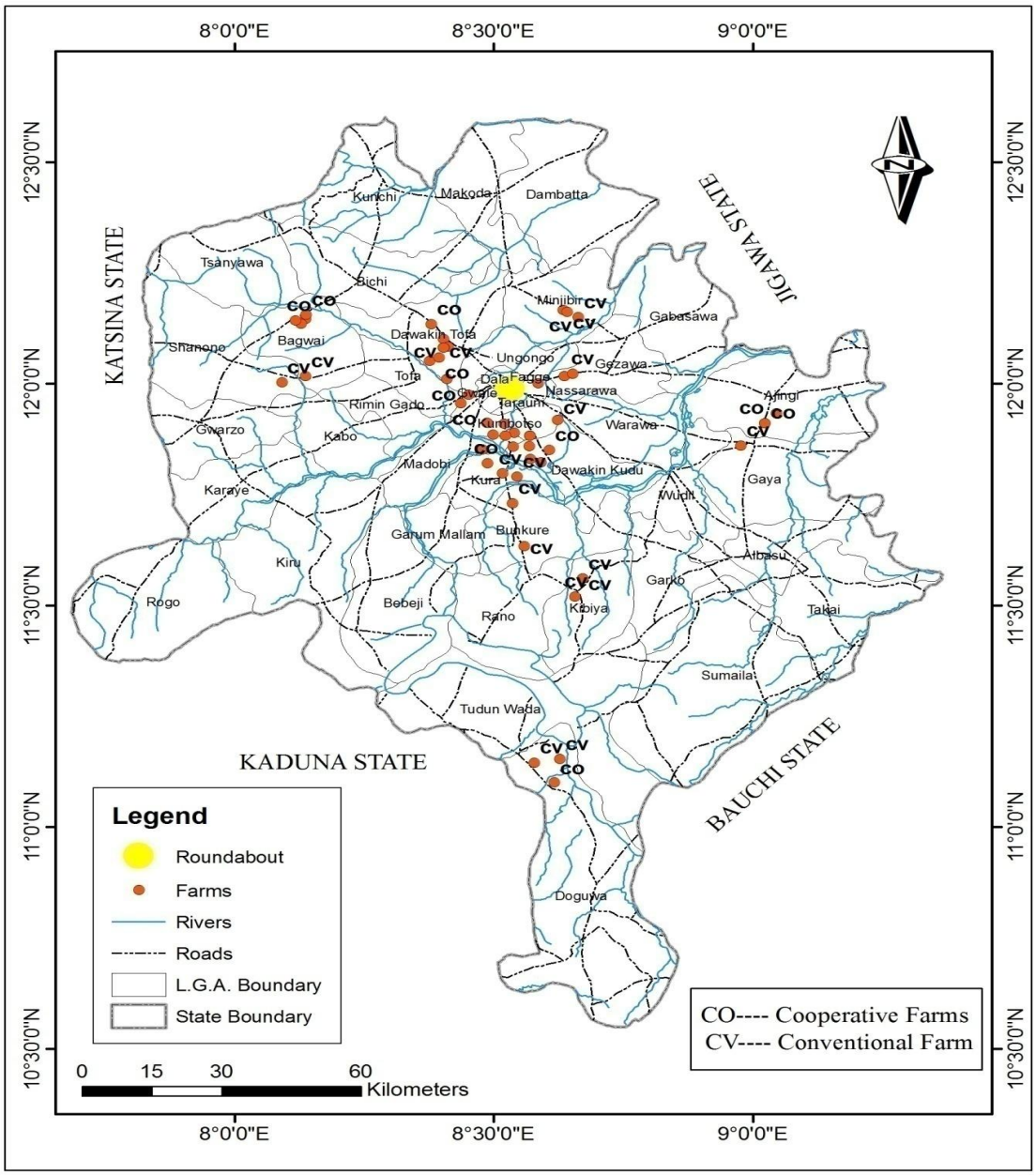


Plate IX: Map of Kano State Showing the Coordinates of the Sampled Farms.